AMANDA TRISTÃO SANTINI

MICROBIOTA INTESTINAL DE *Melipona* spp.: CARACTERIZAÇÃO E IMPACTOS DA PAISAGEM E DO USO DE AGROQUÍMICOS

VIÇOSA – MINAS GERAIS
2024

AMANDA TRISTÃO SANTINI

MICROBIOTA INTESTINAL DE *Melipona* spp.: CARACTERIZAÇÃO E IMPACTOS DA PAISAGEM E DO USO DE AGROQUÍMICOS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Doctor Scientiae*.

Orientador: Cynthia Canêdo da Silva

Coorientadores: Helder Canto Resende
Weyder Cristiano Santana

Colaborador: Alan Emanuel Silva Cerqueira

VIÇOSA – MINAS GERAIS
2024

Ficha catalográfica elaborada pela Biblioteca Central da Universidade Federal de Viçosa – Campus Viçosa

T

S235m
2024

Santini, Amanda Tristão, 1994-
Microbiota intestinal de *Melipona* spp.: caracterização e impactos da paisagem e do uso de agroquímicos / Amanda Tristão Santini. - Viçosa, MG, 2024.
1 tese eletrônica (86 f.): il. (algumas color.).

Texto em português e inglês.
Inclui apêndices.
Orientador: Cynthia Canêdo da Silva.
Tese (doutorado) - Universidade Federal de Viçosa, Departamento de Microbiologia, 2024.
Inclui bibliografia.
DOI: https://doi.org/10.47328/ufvbbt.2024.393
Modo de acesso: World Wide Web.

1. Abelhas sem ferrão. 2. Microbiota. 3. Microbioma gastrointestinal. 4. Dimetoato. 5. Simbiose. I. Silva, Cynthia Canêdo da, 1978-. II. Universidade Federal de Viçosa. Departamento de Microbiologia. Programa de Pós-Graduação em Microbiologia Agrícola. III. Título.

CDD 22. ed. 595.799

Bibliotecário(a) responsável: Alice Regina Pinto Pires CRB-6/2523

AMANDA TRISTÃO SANTINI

MICROBIOTA INTESTINAL DE *Melipona* spp.: CARACTERIZAÇÃO E IMPACTOS DA PAISAGEM E DO USO DE AGROQUÍMICOS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Doctor Scientiae*.

APROVADA: 24 de abril de 2024.

gov.br Documento assinado digitalmente
AMANDA TRISTAO SANTINI
Data: 26/07/2024 09:54:47-0300
Verifique em https://validar.iti.gov.br

_____
Amanda Tristão Santini
Autora

gov.br Documento assinado digitalmente
CYNTHIA CANEDO DA SILVA
Data: 13/08/2024 09:43:07-0300
Verifique em https://validar.iti.gov.br

_____
Cynthia Canêdo da Silva
Orientadora

AGRADECIMENTOS

À Universidade Federal de Viçosa, em particular ao Departamento de Microbiologia, pela oportunidade de realização deste estudo.

À minha orientadora, Professora Cynthia Canêdo da Silva, por todos os ensinamentos, orientação e apoio dados.

Aos Professores Helder Canto Resende e Weyder Cristiano Santana pelo auxílio, orientação e sugestões que contribuíram para a realização deste trabalho.

Ao Professor José Eduardo Serrão pelos ensinamentos e por me permitir utilizar o seu laboratório para treinar técnicas de dissecção de abelhas.

À University of Texas at Austin, em especial à Prof. Nancy Moran pelo acolhimento, ensinamentos e sugestões que contribuíram para a realização deste trabalho.

Ao amigo e colaborador, Dr. Alan Emanuel Silva Cerqueira por todos os ensinamentos, conselhos e apoio durante a minha trajetória no LAMAP e na UT-Austin.

Aos colegas de laboratório da UT – Austin, em especial ao Dr. Erick Motta, Dra. Jo Anne Holley e Dr. Korin Jones pelos ensinamentos e discussões que contribuíram para a análise de dados e escrita da tese.

À minha família, Paulo, Rosilda, Paula e Arthur, por me incentivarem, não largarem a minha mão e nunca me deixarem desistir.

Ao Dr. Tobias Wolf por estar ao meu lado durante todo o meu período em Austin e me dar todo o suporte emocional para concretizar o trabalho.

À Lígia Francisco pelo acolhimento em Austin e pela amizade, incentivo e auxílio durante esse período.

Aos amigos Alex, Wiane, Lutécia, Letícia, Nicolly, Helena e Paula pela amizade, convívio, acolhimento e por todos os momentos de descontração e trabalho.

Às colegas de trabalho, Evelyn Rodrigues, Ana Clara Metri e Stella Braathen por todo auxílio e trabalho desenvolvido durante a realização deste projeto.

Às agências de fomento, FAPEMIG pelo apoio financeiro para realização deste projeto e CNPq pelas bolsas de estudos concedidas.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001.

RESUMO

SANTINI, Amanda Tristão, D.Sc., Universidade Federal de Viçosa, abril de 2024. **Microbiota intestinal de *Melipona* spp.: caracterização e impactos da paisagem e do uso de agroquímicos.** Orientadora: Cynthia Canêdo da Silva. Coorientadores: Helder Canto Resende e Weyder Cristiano Santana.

A microbiota intestinal desempenha um papel fundamental na preservação da saúde de abelhas. As abelhas eusociais corbiculadas (Apini, Bombini e Meliponini) apresentam uma microbiota densa e relativamente simples, entretanto pouco se conhece sobre a composição e papel da microbiota de *Melipona*. Além disso, são escassos e necessários estudos que analisem o impacto de diferentes paisagens e do uso de agroquímicos na microbiota intestinal das abelhas sem ferrão, visando o desenvolvimento de novas estratégias de preservação. Nesse contexto, o presente trabalho teve como objetivos analisar a composição microbiana intestinal de abelhas do gênero *Melipona* coletadas em diferentes regiões do Brasil, a fim de caracterizar a microbiota *core* dessas abelhas e elucidá-la ao longo do trato digestório de *Melipona quadrifasciata*. Adicionalmente, analisar os efeitos da paisagem na microbiota de *M. capixaba,* e os efeitos de doses subletais de dimetoato na microbiota intestinal de *M. quadrifasciata* e *M. mondury*. O trabalho foi dividido em três capítulos. No primeiro, a diversidade microbiana intestinal em *Melipona* spp. coletadas em diversos estados brasileiros, tiveram o DNA intestinal extraído e sequenciado para o gene 16S rRNA, e a caracterização de novos simbiontes em diferentes partes do intestino de *M. quadrifasciata* foram abordadas. A microbiota *core* de *Melipona* spp. incluiu *Bifidobacterium*, *Lactobacillus*, *Apilactobacillus*, *Floricoccus* e *Bombella*. Dentre eles, *Apilactobacillus* e *Bombella* dominaram no papo, enquanto o ventrículo foi dominado por *Apilactobacillus* e *Lactobacillus*. Foi confirmada a ausência de *Snodgrassella* e *Gilliamella* no íleo, no qual verificou-se um novo simbionte filogeneticamente próximo a *Floricoccus*, bem como a presença de *Bifidobacterium*, Lactobacillaceae e *Bombella*. O reto foi dominado por *Bifidobacterium* e *Lactobacillus*. No segundo capítulo, abordou-se a influência da paisagem e da sazonalidade na microbiota intestinal de *M. capixaba*. As abelhas foram coletadas em áreas urbanas, naturais, agrícolas e de agricultura orgânica, ao final do verão e do inverno. O DNA intestinal foi extraído e o gene 16S rRNA sequenciado. A microbiota das abelhas de áreas urbanas diferiu significativamente da microbiota das abelhas coletadas em outras áreas. Abelhas coletadas no verão/2023 também apresentaram uma composição microbiana diferente daquelas coletadas no verão/2022. Entretanto, mais estudos são necessários com um maior número de amostras para elucidar os efeitos da sazonalidade na microbiota de *M. capixaba*. No terceiro capítulo, foram discutidos os efeitos de doses subletais de dimetoato na microbiota intestinal de *M. quadrifasciata* e *M. mondury*. Os grupos de abelhas que apresentaram uma taxa de sobrevivência acima de 65% tiveram seus intestinos extraídos e sequenciados para a região 16S rRNA. Observou-se que as doses subletais de dimetoato não impactaram significativamente a microbiota intestinal de ambas as abelhas testadas. Porém, as abelhas participantes do experimento tiveram uma composição microbiana dissimilar das abelhas do grupo controle de campo, indicando um possível efeito das condições a que foram expostas em laboratório.

Palavras-chave: Meliponini; Microbiota; Diversidade microbiana; Dimetoato; Simbiontes; Intestino.

ABSTRACT

SANTINI, Amanda Tristão, D.Sc., Universidade Federal de Viçosa, April, 2024. **Gut microbiota of *Melipona* spp.: characterization and impacts of the landscape and agrochemicals.** Adviser: Cynthia Canêdo da Silva. Co-advisers: Helder Canto Resende e Weyder Cristiano Santana.

The symbiotic relationship between microorganisms and hosts is essential for the maintenance of species and ecosystems. In the case of animal hosts, specifically insects, the gut microbiota plays a fundamental role in preserving the health of these invertebrates, from their development to protection against infections and detoxification of the organism. Eusocial corbiculate bees (Apini, Bombini, and Meliponini) have a dense and relatively simple microbiota that performs various functions in maintaining the health of these insects, such as pollen digestion and detoxification, nectar fermentation, protection against pathogens, and detoxification of these animals from chemicals commonly used in agriculture. Among corbiculate bees, stingless bees stand out as the main pollinators of native species in tropical and subtropical ecosystems, constituting the most diverse group among eusocial bees. In recent decades, these bees have faced numerous challenges contributing to the decline of their populations, such as climate change, indiscriminate use of agrochemicals, and anthropogenic alterations in their natural habitat. Little is known about the composition, relevance, and role of the microbiota of *Melipona* in the face of challenges faced by these bees. Currently, it is known that *Melipona* lacks two main symbionts present in other bees, *Gilliamella* and *Snodgrassella*, but still lacks information on the acquisition of new symbionts and their role for the host. Additionally, studies analyzing the impact of different environments and the use of agrochemicals on the gut microbiota of stingless bees are scarce and extremely necessary to develop new preservation strategies for these insects. In this context, this study aimed to analyze the gut microbial composition of *Melipona* collected in different regions of Brazil to characterize the core microbiota of these bees and investigate the different parts of the gut of *Melipona quadrifasciata anthidioides* to elucidate the distribution of microorganisms along the digestive tract of these insects. Additionally, the effects of landscape on the microbiota of *M. capixaba* and the effects of sublethal doses of dimethoate on the gut microbiota of *M. quadrifasciata* and *M. mondury* were analyzed. To achieve these objectives, the study was divided into three chapters. In the first chapter, the gut microbial diversity in *Melipona* spp. and the characterization of new symbionts in different gut parts of *Melipona quadrifasciata anthidioides* were addressed. Different bee species were collected in various Brazilian states, with gut DNA being extracted, sequenced for the 16S rRNA gene, and analyzed together with previously published data. Subsequently, the gut of *M. quadrifasciata anthidioides* was sectioned, and each part was sequenced for the 16S rRNA gene. The core microbiota of *Melipona* spp. included *Bifidobacterium*, *Lactobacillus*, *Apilactobacillus*, *Floricoccus*, and *Bombella*. Among them, *Apilactobacillus* and *Bombella* dominated the crop, while the venticulus was dominated by *Apilactobacillus* and *Lactobacillus*. The absence of *Snodgrassella* and *Gilliamella* in the ileum of these bees was confirmed, which contained a new symbiont phylogenetically close to *Floricoccus*, as well as the presence of *Bifidobacterium*, Lactobacillaceae, and *Bombella* in this gut region. The rectum was dominated by *Bifidobacterium* and *Lactobacillus*. In the second chapter, the influence of landscape and seasonality on the gut microbiota of *M. capixaba* was addressed.

Bees were collected in urban, natural, agricultural, and organic farming areas, at the end of the hot and rainy season (summer) and the cold and dry season (winter). Bee gut DNA was extracted and sequenced for the 16S rRNA region. The microbiota of bees collected in urban areas differed significantly from the microbiota of bees collected in other areas. Bees collected at the end of the summer of 2023 also had a different microbial composition from those collected in the summer of 2022 and winter of 2022. Further studies with a larger number of samples are needed to elucidate the effects of seasonality on the microbiota of these bees. In the third chapter, the effects of sublethal doses of dimethoate on the gut microbiota of *M. quadrifasciata* and *M. mondury* were analyzed. Bee groups with a survival rate above 65% had their gut extracted and sequenced for the 16S rRNA region. It was observed that sublethal doses of dimethoate did not significantly impact the microbiota of both tested bees. However, bees participating in the experiment had a dissimilar microbial composition from the field control bees, which were bees sequenced shortly after being removed from the hives, indicating a possible effect of the conditions they were exposed to in the laboratory.

Keywords: Meliponini; Microbiota; Microbial diversity; Dimethoate; Symbiont; Gut.

LISTA DE FIGURAS

Capítulo 1

Figure 1. Mean relative abundance of gut bacterial genera in *Melipona* populations classified using SILVA database..... 26

Figure 2. Microbial community of gut regions of *M. quadrifasciata anthidioides*..... 27

S1 Figure. Most abundant families in *Melipona* spp. gut microbiota..... 37

S2 Figure. Most abundant genera in *Melipona* spp. gut microbiota..... 38

S3 Figure. Phylogenetic trees of the most abundant ASVs (including the 11 core ASVs) found in *Melipona* bee populations..... 39

Capítulo 2

Figure 1. Collection sites of *M. capixaba* samples in Espirito Santo state, Brazil..... 43

Figure 2. Bacterial alpha diversity of the gut microbiota of *M. capixaba* collected in different landscapes..... 46

Figure 3. Mean relative abundance (>0.001) of bacterial genera present in the gut of *M. capixaba* collected in different landscapes and seasons 47

Figure 4. Mean relative abundance (>0.001) of the 20 most abundant ASVs in *M. capixaba* collected in different landscapes and seasons 48

Figure 5. Venn diagram of ASVs present in the bee gut microbiota of *M. capixaba* across environments..... 49

Figure 6. NMDS based on ASV relative abundance (Bray-Curtis dissimilarity) in *M. capixaba* sampled in different landscapes and seasons..... 50

Figure 7. Bacterial alpha diversity of the gut microbiota of *M. capixaba* collected in different landscapes and seasons..... 51

Capítulo 3

Figure 1. Kaplan-Meier survival curves of *Melipona* bees orally exposed for 6 h to dimethoate..... 66

Figure 2. Beta diversity of *Melipona* orally exposed to dimethoate..... 69

Figure 3. Mean relative abundance of Bifidobacteriaceae and Acetobacteraceae of *Melipona* exposed to dimethoate and controls..... 70

Figure 3. Mean relative abundance of bacterial genera (>0.001) in the gut microbiota of *Melipona* bees..... 71

Figure 4. Mean relative abundance (>0.001) of most abundant ASVs in *Melipona* exposed to dimethoate and controls..... 73

S1 Figure. Bacterial alpha diversity expressed by Shannon Index and Richness of *Melipona* orally exposed to dimethoate..... 84

S2 Figure. Mean relative abundance of bacterial families in the gut microbiota of *Melipona* bees..... 85

LISTA DE TABELAS

Capítulo 1

S1 Table. Information of collection, species name and source of the *Melipona* samples analyzed in the present work……..…….. 32

S2 Table. PERMANOVA based on the Bray-Curtis dissimilarity matrix comparing the differences in the microbial community composition between the gut regions of *M. quadrifasciata anthidioides*…..……..…… 33

S3 Table. GenBank sequences used for analysis…….. 34

Capítulo 2

Table 1. Pairwise comparison of the gut bacterial community of *Melipona capixaba* in different landscapes and seasons……..……..…….. 46

Capítulo 3

S1 Table. Pairwise comparison of the gut bacterial community of *Melipona* exposed to sublethal doses of dimethoate……..……... 82

S2 Table. Pairwise Kruskal-Wallis test with post hoc Dunn test ($p < 0.05$) for the mean relative abundance of Bifidobacteriaceae and Acetobacteraceae…….. 83

SUMÁRIO

1. INTRODUÇÃO GERAL 13
2. REFERENCIAL TEÓRICO 14
3. OBJETIVOS 18
REFERÊNCIAS 19
CAPÍTULO 1 21
THE UNIQUE MICROBIOME OF *MELIPONA* STINGLESS BEES: UNRAVELING THE CORE GUT BACTERIA AND PUTATIVE NEW SYMBIONTS ACROSS DIFFERENT GUT REGIONS 21
INTRODUCTION 22
METHODOLOGY 23
RESULTS 24
DISCUSSION 27
REFERENCES 28
SUPPLEMENTARY MATERIAL 31
CAPÍTULO 2 40
SEASONAL AND LANDSCAPE INFLUENCES ON THE GUT MICROBIOTA OF THE ENDANGERED *MELIPONA CAPIXABA* BEES 40
INTRODUCTION 40
METHODOLOGY 42
Sample collection and season conditions 42
Agrochemical analysis 44
DNA extraction, 16S rRNA gene sequencing, and data processing 44
RESULTS 45
Influence of the landscape in the bee gut microbiota 45
The gut microbial community in M. capixaba varies seasonally 49
DISCUSSION 51
CONCLUSION 53
REFERENCES 55
SUPPLEMENTARY MATERIAL 59
CAPÍTULO 3 61
DIMETHOATE IS HIGHLY TOXIC FOR *MELIPONA* BEES BUT DOES NOT AFFECT THEIR GUT MICROBIOME IN SUBLETHAL DOSES 61
INTRODUCTION 61
METHODOLOGY 63
Insects and mortality bioassay 63
DNA extraction, 16S rRNA gene sequencing, and data processing 65
RESULTS 66
Bacterial diversity in the gut of Melipona bees treated with dimethoate 67

DISCUSSION 72
CONCLUSION 74
REFERENCES 75
SUPPLEMENTARY MATERIAL 80
4. CONCLUSÃO GERAL 84

1. INTRODUÇÃO GERAL

A relação simbiótica entre insetos e microrganismos é essencial para a manutenção das espécies e ecossistemas. Múltiplos microrganismos habitam o sistema digestório desses animais, melhorando a saúde geral do hospedeiro e mantendo o seu fitness. Dessa forma, a microbiota de abelhas tem um papel fundamental desde o desenvolvimento desses insetos até o fim da sua vida.

A maioria das abelhas corbiculadas (Apini, Bombini e Meliponini) apresenta uma microbiota core representada por quatro principais gêneros: *Lactobacillus, Bifidobacterium, Snodgrassella* e *Gilliamella*. Esses microrganismos atuam na digestão do pólen e do mel, na melhoria da resposta imune e proteção contra patógenos, bem como na sinalização hormonal do organismo. Além da microbiota core, essas abelhas apresentam uma microbiota ambiental transiente, que varia de acordo com a idade, pressões ambientais e espécie. Apesar de apresentarem uma microbiota parecida com à de *Apis mellifera* e *Bombus* spp., um recente trabalho do nosso grupo de pesquisa com abelhas do gênero *Melipona* observou a ausência de simbiontes importante para outras abelhas corbiculadas, um fato intrigante e ainda pouco explorado.

A maioria dos estudos relacionados à microbiota de abelhas concentra-se em abelhas do gênero *Apis* e *Bombus*, havendo uma escassez em relação às outras espécies existentes. Estima-se que a diversidade global desses insetos ultrapassa 20.000 espécies e, as abelhas sociais representam cerca de 4% desse total. Ainda, os meliponíneos são os principais responsáveis pela polinização e manutenção dos ecossistemas nativos nas regiões tropicais e subtropicais; sendo, ainda, fonte de renda para muitas famílias meliponicultoras. Dessa forma, o presente trabalho visa analisar a microbiota de abelhas do gênero *Melipona*, bem como os impactos da paisagem e uso de agroquímicos sobre a comunidade microbiana desses insetos.

2. REFERENCIAL TEÓRICO

O sucesso evolutivo de muitos insetos está diretamente relacionado à sua associação simbiótica com microrganismos (DOUGLAS, 2015). Múltiplas espécies habitam o sistema digestório desses animais, trazendo benefícios desde o aumento da oviposição, longevidade, diminuição do período larval, aumento da resiliência aos distúrbios ambientais ou mudanças no comportamento do hospedeiro (AKAMI et al., 2019; ENGEL; MORAN, 2013; GOULD et al., 2018; LIBERTI; ENGEL, 2020). Esses benefícios acontecem porque a maioria dos animais não apresenta enzimas necessárias para degradar todos os nutrientes presentes na sua alimentação, o que é digerido pela microbiota intestinal que, por sua vez, libera produtos da fermentação que são extremamente importantes para o metabolismo do hospedeiro (LEE; HASE, 2014).

Nesse sentido, a microbiota das abelhas tem um papel fundamental desde o desenvolvimento desses insetos até o fim da sua vida. Abelhas do gênero *Apis* e *Bombus* apresentam comunidades microbianas que englobam, principalmente bactérias do gênero *Lactobacillus* (Firm-5 e Firm-4), *Bifidobacterium, Snodgrassella alvi* e *Gilliamella apicola* (BONILLA-ROSSO; ENGEL, 2018; MORAN, 2015). Esses microrganismos atuam na digestão do pólen, que contém diversos carboidratos, proteínas, lipídeos e outros micronutrientes, e do mel (RICIGLIANO et al., 2017). ZHENG et al. (2016) relataram que isolados de *G. apicola* secretam pectinases e foram capazes de fermentar açúcares tóxicos para as abelhas, como pectina, manose, xilose e arabinose, atuando na detoxificação do pólen. Dessa forma, a microbiota está ainda associada ao ganho de peso desses insetos e sinalização hormonal (ZHENG et al., 2017).

Os microrganismos também estão associados a outros parâmetros da alimentação das abelhas. Para a fermentação do pólen e fabricação do *bee bread*, bem como para a transformação do néctar em mel, as abelhas inoculam bactérias láticas que também protegem o seu alimento da deterioração dentro da colmeia (KHAN et al., 2020). As *A. mellifera* coletam *pellets* de *Cladosporium* em áreas de escassez de alimentos e utilizam esse microrganismo como fonte de nutrientes (MODRO et al., 2009). Espécies de abelhas sem ferrão, como *Scaptotrigona depilis*, dependem da ingestão de *Zygosaccharomyces* sp., fungo que cresce dentro das

células de cria sobre o alimento larval, que produz esteróis necessários para o desenvolvimento larval (PALUDO et al., 2019).

Além disso, a microbiota *core* das abelhas atua na resposta imune do hospedeiro. *S. alvi* coloniza o íleo de *A. mellifera* e, em associação com *G. apicola*, formam um biofilme denso que funciona como uma barreira contra patógenos (MARTINSON; MOY; MORAN, 2012). *S. alvi*, induz o aumento da expressão de genes relacionados aos peptídeos antimicrobianos, melhorando a resistência a patógenos (HORAK; LEONARD; MORAN, 2020), promovendo também uma resposta imune que confere maior resistência às infecções virais (KATSNELSON, 2015). Já em abelhas do gênero *Bombus*, a presença na microbiota intestinal reduz significativamente a infecção pelo parasita *Crithidia bombi* (KOCH; SCHMID-HEMPEL, 2011). Os microrganismos contribuem, ainda, com a saúde das abelhas através da presença de genes de bombas de efluxo, promovendo a desintoxicação desses animais de compostos químicos comumente encontrados na agricultura, como agroquímicos (ENGEL; MARTINSON; MORAN, 2012). *Streptomyces* sp. e *Micromonospora* sp. isoladas de *M. rufiventris* produzem compostos capazes de inibir o crescimento de *Paenibacillus larvae*, bactéria causadora da doença *American foulbrood* em *A. mellifera* (RODRÍGUEZ-HERNÁNDEZ et al., 2019).

Entre as abelhas corbiculadas, apesar da ocorrência de alguns microrganismos em comum em determinadas espécies, a microbiota de cada hospedeiro difere quando comparado entre as espécies de *A. mellifera*, *A. cerana*, abelhas sem ferrão e *Bombus* sp. Esses animais apresentam uma microbiota core específica e uma microbiota de origem ambiental, adquirida durante o forrageio (KWONG et al., 2017). Algumas bactérias parecem ser estritamente ligadas a certos hospedeiros. A microbiota de *A. mellifera* pode diferir de acordo com o clima, apresentando uma menor diversidade alfa, porém com um aumento nos níveis de *Bartonella* e *Commensalibacter* (KEŠNEROVÁ et al., 2020). As abelhas sem ferrão do gênero *Melipona* não apresentam bactérias essenciais na microbiota core de *A. mellifera*, *Bombus* spp., como *Snograssella* e *Gilliamella*, o que sugere que essas bactérias foram perdidas durante a evolução dessas abelhas (CERQUEIRA et al., 2021; HAAG et al., 2022; SARTON-LOHÉAC et al., 2023).

Considerando a importância ambiental e social das abelhas, e sendo sua biodiversidade de espécies e comportamentos diretamente relacionados ao

ecossistema em que vivem, estudos visando entender a microbiota intestinal desses animais e sua relação com o ambiente são fundamentais para a manutenção dos ecossistemas. Nesse sentido, as abelhas sem ferrão (Apidae, Meliponini) são consideradas as principais polinizadoras de espécies nativas nos ecossistemas tropicais e subtropicais, sendo importantes também para a polinização de plantas economicamente significativas para a agricultura local (CHAM et al., 2019). Por outro lado, a meliponicultura tem importância socioeconômica para diversas comunidades brasileiras. Os meliponicultores se beneficiam da venda dos produtos dessas abelhas, bem como das colmeias, o que ajuda na manutenção das espécies e é fonte de renda para diversas famílias (JAFFÉ et al., 2015). A criação dessas abelhas, por serem menos agressivas e perigosas do que as abelhas africanizadas, tem sido comum inclusive em áreas urbanas no Brasil.

Entretanto, nas últimas décadas, essas abelhas vêm sofrendo desafios que levam ao declínio de suas populações como o uso de agroquímicos, novos patógenos, mudanças climáticas e alterações antropogênicas no seu habitat natural, o que diminui a disponibilidade de alimentos e locais de nidificação (GIANNINI et al., 2020; GOULSON et al., 2015; GUIMARÃES-CESTARO et al., 2020). As populações remanescentes dessas abelhas estão vulneráveis à endogamia, o que aumenta a produção de machos diploides que são estéreis e inviáveis para a sobrevivência das colônias (CORTOPASSI-LAURINO et al., 2006). A perda da diversidade floral relacionada às ações antrópicas, reduz a disponibilidade e diversidade de pólen e néctar, acarretando um déficit nutricional para as abelhas, o que as torna mais susceptíveis a patógenos (DOLEZAL; TOTH, 2018). Algumas espécies de abelhas nativas sem ferrão são reconhecidas como vulneráveis ou em risco de extinção, incluindo *M. capixaba, M. rufiventris* e *M. scutellaris* (MACHADO et al., 2014).

Ademais, a exposição constante desses insetos aos agroquímicos vem sendo relatada como uma das principais causas das perdas das colmeias. Um dos fenômenos que tem acontecido nesse sentido, em *A. mellifera*, é o chamado transtorno do colapso de colônias (*colony collapse disorder*), relatado nos Estados Unidos e Europa na última década (DAINAT; VANENGESLDORP; NEUMANN, 2012; VANENGELSDORP et al., 2011), mas também observado no Brasil para *M. quadrifasciata* (DÍAZ et al., 2017; HAAG et al., 2022). No caso de abelhas sem ferrão, algumas espécies aparentam ser mais sensíveis aos pesticidas, como *M. scutellaris*

que demonstrou uma sensibilidade maior a abamectina e difenoconazol (BRIGANTE et al., 2021). Pesticidas das classes de neonicotinoides e piretroides apresentam ação direta no comportamento social de *M. quadrifasciata* (BOFF et al., 2018). A contaminação do alimento larval por glifosato e a toxina Cry de *Bacillus thuringiensis* de plantas transgênicas é letal e altera o desenvolvimento de *M. quadrifasciata* (SEIDE et al., 2018). Os compostos usados no meio agrícola podem como o glifosato, mesmo em doses subletais, afetar a microbiota intestinal de *A. mellifera* (MOTTA; RAYMANN; MORAN, 2018).

Sendo assim, estudos que visam à caracterização da microbiota intestinal de abelhas, com ênfase nas espécies nativas brasileiras, são cruciais para uma melhor compreensão do funcionamento geral desses organismos. Ademais, entender o impacto de ações antrópicas, clima e uso de agrotóxicos sobre essa microbiota auxilia na compreensão do impacto indireto de fatores ambientais na saúde geral das colmeias, subsidiando a proposição de práticas de manejo mais eficientes para a preservação dessas espécies.

3. OBJETIVOS

Determinar a estrutura microbiana de abelhas sem ferrão do gênero *Melipona* de diferentes regiões do Brasil e definir a microbiota core de cada parte do intestino de *M. quadrifasciata anthidioides*.

Analisar os efeitos da paisagem e estações do ano sobre a microbiota de *M. capixaba* proveniente do Espírito Santo.

Analisar o efeito de doses subletais de dimetoato na microbiota de *M. quadrifasciata anthidioides* e *M. mondury*.

REFERÊNCIAS

AKAMI, M. et al. Symbiotic bacteria motivate the foraging decision and promote fecundity and survival of *Bactrocera dorsalis* (Diptera: Tephritidae). **BMC Microbiology**, v. 19, n. 1, p. 1–13, 2019.

BOFF, S. et al. Changes in social behavior are induced by pesticide ingestion in a Neotropical stingless bee. **Ecotoxicology and Environmental Safety**, v. 164, n. December 2017, p. 548–553, 2018.

BONILLA-ROSSO, G.; ENGEL, P. Functional roles and metabolic niches in the honeybee gut microbiota. **Current Opinion in Microbiology**, v. 43, p. 69–76, 2018.

BRIGANTE, J. et al. Acute toxicity of the insecticide abamectin and the fungicide difenoconazole (individually and in mixture) to the tropical stingless bee *Melipona scutellaris*. **Ecotoxicology**, v. 30, p. 1872–1879, 2021.

CERQUEIRA, A. E. S. et al. Extinction of anciently associated gut bacterial symbionts in a clade of stingless bees. **The ISME Journal**, p. 2–5, 2021.

CHAM, K. O. et al. Pesticide Exposure Assessment Paradigm for Stingless Bees. **Environmental Entomology**, v. 48, n. 1, p. 36–48, 2019.

CORTOPASSI-LAURINO, M. et al. Global meliponiculture: challenges and opportunities. **Apidologie**, v. 37, n. 2, p. 275–292, 2006.

DAINAT, B.; VANENGESLDORP, D.; NEUMANN, P. Colony collapse disorder in Europe. **Environmental Microbiology Reports**, v. 4, n. 1, p. 123–125, 2012.

DÍAZ, S. et al. Report on the microbiota of *Melipona quadrifasciata* affected by a recurrent disease. **Journal of Invertebrate Pathology**, v. 143, p. 35–39, 2017.

DOLEZAL, A. G.; TOTH, A. L. Feedback between nutrition and disease in honeybee health. **Current Opinion in Insect Science**, v. 26, p. 114–119, 2018.

DOUGLAS, A. E. Multiorganismal insects: Diversity and function of resident microorganisms. **Annual Review of Entomology**, v. 60, p. 17–34, 2015.

ENGEL, P.; MARTINSON, V. G.; MORAN, N. A. Functional diversity within the simple gut microbiota of the honeybee. **PNAS**, v. 109, n. 27, 2012.

ENGEL, P.; MORAN, N. A. The gut microbiota of insects - diversity in structure and function. **FEMS Microbiology Reviews**, v. 37, n. 5, p. 699–735, 2013.

GIANNINI, T. C. et al. Climate change in the Eastern Amazon: crop-pollinator and occurrence-restricted bees are potentially more affected. **Regional Environmental Change**, v. 20, n. 1, 2020.

GOULD, A. L. et al. Microbiome interactions shape host fitness. **Proceedings of the National Academy of Sciences of the United States of America**, v. 115, n. 51, p. E11951–E11960, 2018.

GOULSON, D. et al. Bee declines driven by combined Stress from parasites, pesticides, and lack of flowers. **Science**, v. 347, n. 6229, p. 1–16, 2015.

GUIMARÃES-CESTARO, L. et al. Occurrence of virus, microsporidia, and pesticide residues in three species of stingless bees (Apidae: Meliponini) in the field. **Science of Nature**, v. 107, n. 3, 2020.

HAAG, K. L. et al. Temporal Changes in Gut Microbiota Composition and Pollen Diet Associated with Colony Weakness of a Stingless Bee. **Microbial Ecology**, n. 0123456789, 2022.

HORAK, R. D.; LEONARD, S. P.; MORAN, N. A. Symbionts shape host innate immunity in honeybees. **Proceedings of the Royal Society B**, v. 287, 2020.

JAFFÉ, R. et al. Bees for development: Brazilian survey reveals how to optimize stingless beekeeping. **PLoS ONE**, v. 10, n. 3, p. 1–21, 2015.

KATSNELSON, A. Microbiome: the puzzle in a bee’ s gut. **Nature**, v. 521, n. S56, p. 12526, 2015.
KEŠNEROVÁ, L. et al. Gut microbiota structure differs between honeybees in winter and summer. **ISME Journal**, v. 14, n. 3, p. 801–814, 2020.
KHAN, K. A. et al. Structural diversity and functional variability of gut microbial communities associated with honeybees. **Microbial Pathogenesis**, v. 138, n. September 2019, p. 103793, 2020.
KOCH, H.; SCHMID-HEMPEL, P. Socially transmitted gut microbiota protects bumble bees against an intestinal parasite. **Proceedings of the National Academy of Sciences of the United States of America**, v. 108, n. 48, p. 19288–19292, 2011.
KWONG, W. K. et al. Dynamic microbiome evolution in social bees. **Science Advances**, v. 3, n. March, p. 1–17, 2017.
LEE, W. J.; HASE, K. Gut microbiota-generated metabolites in animal health and disease. **Nature Chemical Biology**, v. 10, n. 6, p. 416–424, 2014.
LIBERTI, J.; ENGEL, P. The gut microbiota — brain axis of insects. **Current Opinion in Insect Science**, v. 39, p. 6–13, 2020.
MACHADO, A. B. M. et al. Panorama Geral dos Invertebrados Terrestres Ameaçados de Extinção. Em: **ICMBio**. [s.l: s.n.]. p. 80–83.
MARTINSON, V. G.; MOY, J.; MORAN, N. A. Establishment of Characteristic Gut Bacteria during Development of. **Applied and Environmental Microbiology**, v. 78, n. 8, p. 2830–2840, 2012.
MODRO, A. F. H. et al. Saprophytic Fungus Collection by Africanized Bees in Brazil. **Neotropical Entomology**, v. 38, n. April, p. 2005–2007, 2009.
MORAN, N. A. Genomics of the honeybee microbiome. **Current Opinion in Insect Science**, v. 10, p. 22–28, 2015.
MOTTA, E. V. S.; RAYMANN, K.; MORAN, N. A. Glyphosate perturbs the gut microbiota of honeybees. **Proceedings of the National Academy of Sciences of the United States of America**, v. 115, n. 41, p. 10305–10310, 2018.
PALUDO, C. R. et al. Microbial community modulates growth of symbiotic fungus required for stingless bee metamorphosis. **PLoS ONE**, v. 14, n. 7, p. 1–12, 2019.
RICIGLIANO, V. A. et al. The impact of pollen consumption on honey bee (*Apis mellifera*) digestive physiology and carbohydrate metabolism. **Insect Biochemistry and Physiology**, p. 1–14, 2017.
RODRÍGUEZ-HERNÁNDEZ, D. et al. Actinobacteria associated with stingless bees biosynthesize bioactive polyketides against bacterial pathogens. **New Journal of Chemistry**, v. 43, n. 25, p. 10109–10117, 2019.
SARTON-LOHÉAC, G. et al. Deep Divergence and Genomic Diversification of Gut Symbionts of Neotropical Stingless Bees. **mBio**, v. 14, n. 2, 25 abr. 2023.
SEIDE, V. E. et al. Glyphosate is lethal and Cry toxins alter the development of the stingless bee *Melipona quadrifasciata*. **Environmental Pollution**, v. 243, p. 1854–1860, 2018.
VANENGELSDORP, D. et al. A survey of managed honey bee colony losses in the USA , fall 2009 to winter 2010. **Journal of Apicultural Research**, v. 50, n. 1, p. 1–10, 2011.
ZHENG, H. et al. Honeybee gut microbiota promotes host weight gain via bacterial metabolism and hormonal signaling. **PNAS**, v. 114, n. 18, p. 4775–4780, 2017.

CAPÍTULO 1

The unique microbiome of *Melipona* stingless bees: unraveling the core gut bacteria and putative new symbionts across different gut regions

Short title: **Unraveling Melipona bee gut microbiome**

Amanda Tristao Santini[1,2*], Alan Emanuel Silva Cerqueira[1,2], Nancy A. Moran[2], Helder Canto Resende[3], Cynthia Canedo da Silva[1]

[1] Department of Microbiology, Federal University of Viçosa, Viçosa, MG 36570-900, Brazil.
[2] Department of Integrative Biology, The University of Texas at Austin, Austin, TX 78712, USA.
[3] Institute of Biological and Health Science, Federal University of Viçosa, Florestal, MG 35690-000, Brazil.

*Corresponding author: amandatsantini@gmail.com

(Artigo submetido à PLOS ONE, encontra-se sob revisão)

Abstract

The gut microbiome of eusocial corbiculate bees, which include honeybees, bumblebees, and stingless bees, consists of anciently associated, host-specific bacteria that are vital for bee health. Two symbionts, *Snodgrassella* and *Gilliamella*, are ubiquitous in honeybees and bumblebees However, their presence varies in the stingless bee clade (Meliponini). They are absent or rare in the diverse and widespread genus *Melipona*, indicating a shift in microbiota composition in this lineage. To determine the core members of the *Melipona* microbiome, we combined newly collected and published data from field-collected individuals of several species. Additionally, we identified the localization of the core microbiota within the gut regions of *Melipona quadrifasciata anthidioides*. The core microbiota of *Melipona* includes members of the genera *Bifidobacterium*, *Lactobacillus*, *Apilactobacillus*, *Floricoccus*, and *Bombella*. Among these, *Apilactobacillus* and *Bombella* dominate the crop, whereas *Apilactobacillus* and other members of the Lactobacillaceae dominate the ventriculus. The ileum lacks *Snodgrassella* or *Gilliamella* but contains a putative new symbiont close to *Floricoccus*, as well as strains of *Bifidobacterium*, Lactobacillaceae (including *Apilactobacillus*), and *Bombella*. The rectum is dominated by *Bifidobacterium* and *Lactobacillus*. In summary, *Melipona* has a unique core microbiota that distinguishes it from honeybees and bumblebees.

Keywords: microbiota, symbiosis, corbiculate bees, *Floricoccus*, microbial diversity.

Introduction

Eusocial corbiculate bees comprise three clades, the honeybees (genus *Apis*), bumblebees *(*genus *Bombus*), and stingless bees (tribe Meliponini) [1]. Their gut microbiomes contain anciently associated, host-specific bacteria that can contribute to bee health [2–4]. In guts of both honeybees and bumblebees, *Snodgrassella* and *Gilliamella* strains dominate in the ileum, while *Bombilactobacillus*, *Lactobacillus* nr. *melliventris*, and *Bifidobacterium* strains dominate in the rectum [4–6]. In the stingless bees, *Snodgrassella* and *Gilliamella* vary in occurrence, having been lost/rare in some clades, including the large Neotropical genus, *Melipona* [3,7–10]. In *Melipona,* the functional roles of *Snodgrassella* and *Gilliamella* have been speculated to have been

replaced by new symbionts [7], including a member of the family Streptococcaceae, close to *Floricoccus* and consistently found in *Melipona* species [7–9].

Here, we inferred the core members of the *Melipona* Illiger, 1806 microbiome by combining newly collected and published data on gut bacterial communities of field-collected individuals of several species. In addition, we determined the localization of the core bacteria to different gut regions within the species *Melipona quadrifasciata anthidioides* Lepeletier, 1836. Our results add to the understanding of the shifts in microbiota structure that have occurred in *Melipona*, including a possible replacement of *Snodgrassella* and *Gilliamella* by new symbionts.

Methodology

The sample collection was authorized by the Brazil Ministry of Environment (SISBIO/ICMBIO authorization number 87892-1). We collected bees from different colonies of 10 *Melipona* populations (i.e. bees from the same species living at the same sampling location) across different locations in Brazil. The populations consisted of two species identified by comparison with known specimens and/or taxonomic keys [11] and five morphotypes whose identification was not confirmed (referred to as "*Melipona* cf. = *conferatum*"). All bee samples collected for this work are listed as “New” in the “Source” tab of Supplementary S1 Table. The number of colonies collected varied based on availability in each location. *M. quadrifasciata anthidiodes* was selected to evaluate the bacterial diversity of each gut part due to its availability in the region. For this species, we collected bees from 3 different colonies in Viçosa – MG, Brazil.

We collected 10 bees from each box entrance, except for the *M. quadrifasciata anthidioides* samples. These bees were then placed in sterile tubes containing 95% ethanol. Out of these collected bees, 5 bees from each box were dissected using sterile forceps with a stereoscopic microscope. Each sample comprised a pool of the 5 guts from the dissected bees. To assess the microbial diversity in each gut section of *M. quadrifasciata anthidiodes*, we collected three bees from each of the three boxes sampled. The gut was divided into four parts: crop, ventriculus, ileum, and rectum, and each part was treated as a separate sample. The total DNA was then extracted using the NucleoSpin soil kit (Macherey-Nagel), preceded by a proteinase K treatment for 2 hours at 56ºC [7]. After extraction, the DNA was submitted for 250 bp paired-end amplicon sequencing at Novogene Corporation Inc (Sacramento, CA, USA) using an

Illumina NovaSeq 6000 System. The primer pair 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACNNGGGTATCTAAT) was used to target the 16S rRNA V3-V4 regions. The data were processed together with previously published data (SRA accession #PRJNA678404) [7] using the DADA2 package (version 1.28) [12] in R 4.3.1, following the pipeline available at https://benjjneb.github.io/dada2/tutorial.html. The taxonomy was assigned to ASVs using a trained SILVA database (version 138.1 from November 2020) for bacteria. For data analysis, we used the R package "mctoolsr" version 0.1.1.9 (available at https://github.com/leffj/mctoolsr), “vegan” version 2.6-4 [13], and "ggplot2" version 3.4.2 [14].

Furthermore, the most abundant and core ASVs were submitted to BLASTN similarity searches against GenBank at NCBI Reference Sequence Database at which we could identify and download sequences from isolates aligned to them. Downloaded sequences were aligned using MAFFT 7 [15], and the Maximum Likelihood phylogenetic tree was made with a bootstrap of 1000 replications using IQ-TREE 2 [16]. By this approach we could determine the possible origin of dominant ASVs in *Melipona*. The phylogenetic trees were rooted according to the outgroups: (A) *Fructilactobacillus fructivorans*, (B) *Amylolactobacillus amylophilus,* (C) *Lactiplantibacillus plantarum*, (D) *Bombiscardovia coagulans*, (E) *Granulibacter bethesdensis* (S3 Table, S3 Fig.).

Results

Melipona bees possess a consistent microbiota mainly composed of species of Acetobacteraceae, Bifidobacteriaceae, Lactobacillaceae, and Streptococcaceae (Fig. S1). Genera present in all individuals include *Apilactobacillus*, *Bifidobacterium*, *Bombella, Commensalibacter*, *Floricoccus*, *Lactobacillus*, and *Neokomagataea* (Fig. 1, S2 Fig). A few samples contain other environmental genera, such as *Prevotella*, *Rosenbergiella*, and *Weissella*.

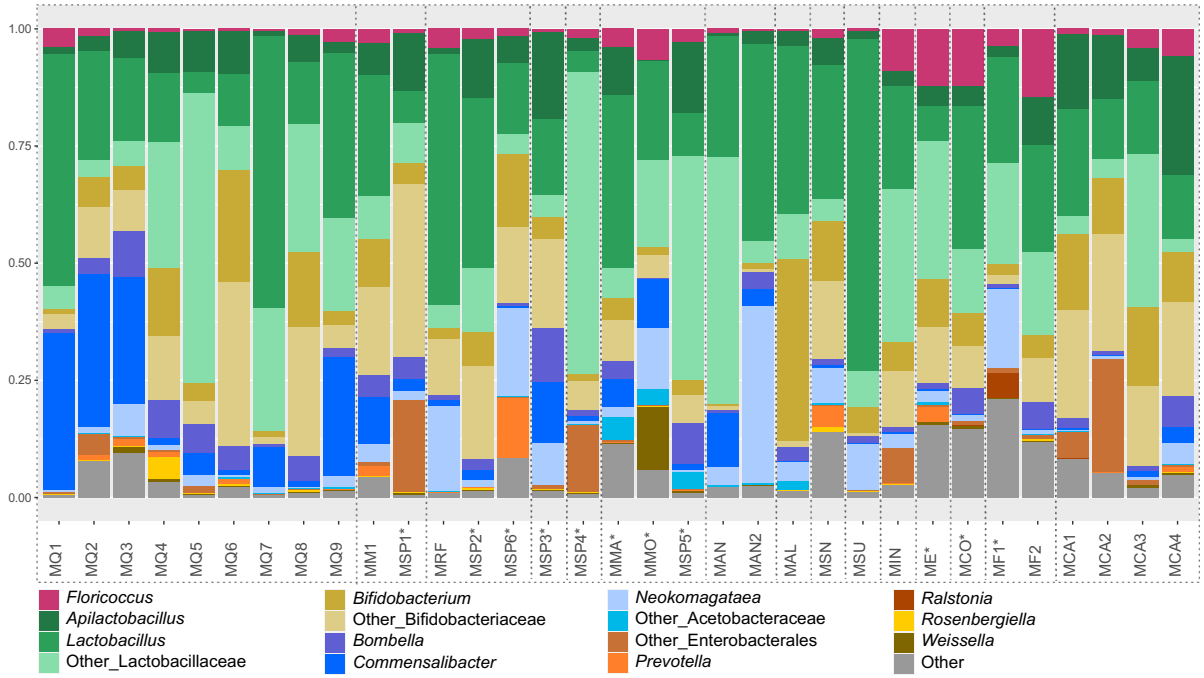


Fig 1. Mean relative abundance of gut bacterial genera in *Melipona* populations classified using SILVA database. Each column represents 5 pooled bees from a population. 'Other_Lactobacillaceae' refers to bacteria assigned to Lactobacillaceae that could not be identified at the genus level. Similarly, 'Other_Acetobacteraceae' refers to bacteria assigned to Acetobacteraceae that could not be identified at the genus level. 'Other_Enterobacterales' refers to bacteria only identified at the order level. 'Other' are bacteria in lower abundance. See Table S1 for population and collection information. Populations grouped by the dotted lines are considered from the same *Melipona* species. *Species whose identification was not confirmed.

Concerning the *M. quadrifasciata* (Mqa) gut regions the NMDS based on ASV relative abundances separated the samples by region but not by source colony (Fig. 2A), and PERMANOVA analysis showed significant differences among gut regions (S2 Table).

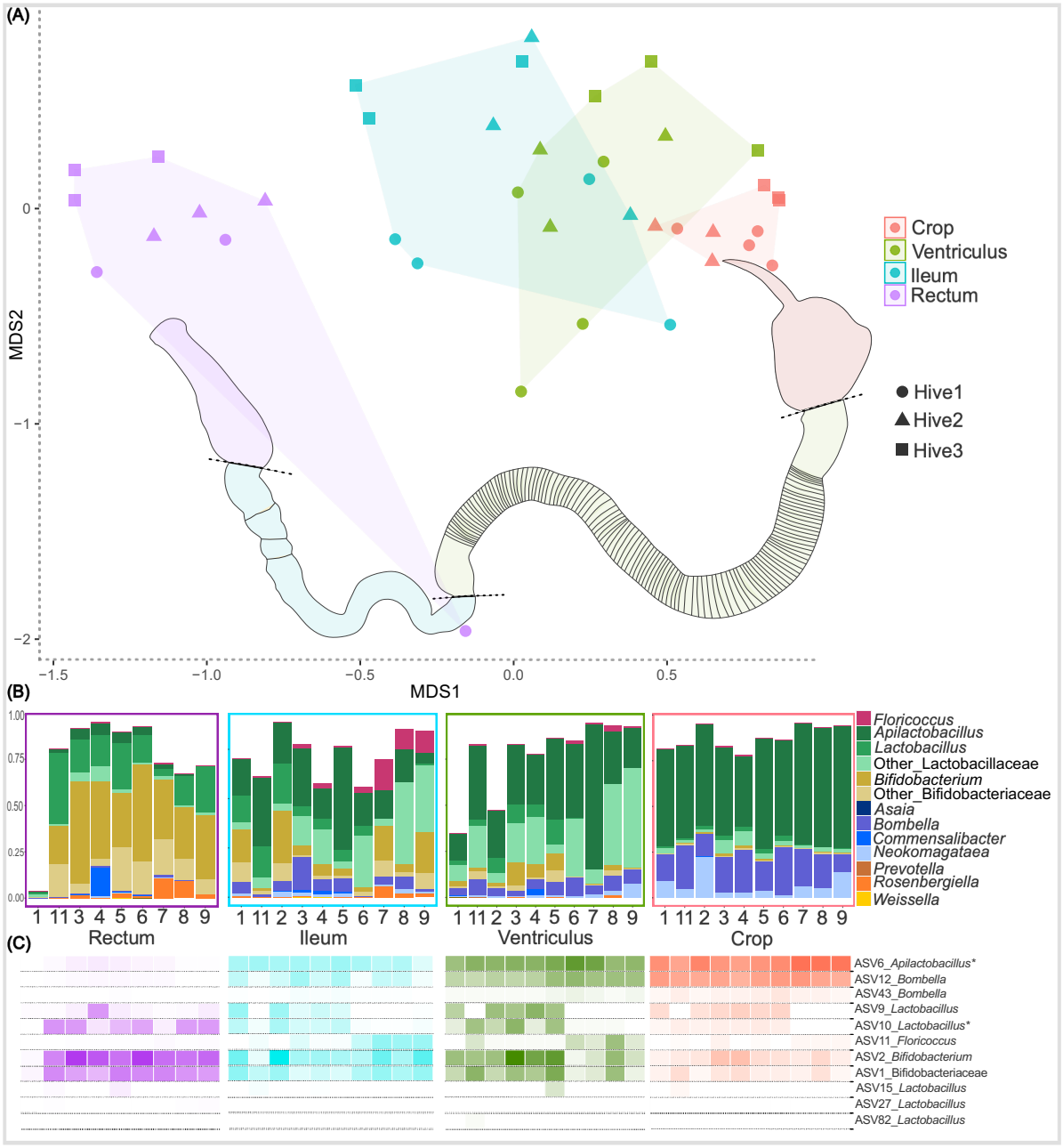


Fig 2. Microbial community of gut regions of *M. quadrifasciata anthidioides*. (A) NMDS based on ASV relative abundance (Bray-Curtis dissimilarity) in gut regions of bees from three hives. (B) Relative abundance of core genera, classified using SILVA database, in each gut part. (C) Heatmap of *Melipona* core ASVs in each gut part classified using SILVA database. *[1]ASV6 was classified as *Apilactobacillus* using SILVA database but formed a clade with *Nicoliella* using Genbank Nucleotide Database sequences (see Fig. S3). *[2]ASV10 was classified as *Floricoccus* using SILVA database but formed a clade with yet undescribed Streptococacceae isolates close to *Floricoccus* using Genbank Nucleotide Database sequences (see S3 Fig).

The genera that are more abundant in *Melipona* generally compose more than 70% of the community in individual gut regions. However, gut regions have distinct compositions. The crop is dominated by *Apilactobacillus*, *Bombella*, and *Neokomagataeae* (Fig. 2B); the ventriculus by *Apilactobacillus*, other Lactobacillaceae, *Bombella,* and Bifidobacteriaceae; the ileum by Lactobacillaceae (including *Apilactobacillus* and *Lactobacillus*), Bifidobacteriaceae (including *Bifidobacterium*), *Bombella,* and *Floricoccus*; and, the rectum by Bifidobacteriaceae (including *Bifidobacterium*) and Lactobacillaceae (including *Apilactobacillus* and *Lactobacillus*). Interestingly, a sequential decrease is observed for *Apilactobacillus* from the crop to the rectum. *Bombella* is also more abundant in the crop compared to ventriculus and ileum. Alternatively, an opposite trend is observed for *Bifidobacterium* and other Bifidobacteriaceae, which increase from the ventriculus to the rectum, where they are the main colonizers along with *Lactobacillus*.

Of the total 1690 ASVs in the samples, 11 ASVs are present in all species of *Melipona* and are considered the core microbiota members (Fig. 2C). These 11 ASVs are related to *Bifidobacterium*, *Bombella*, *Floricoccus, Lactobacillus*, and *Apilactobacillus*. We created phylogenies for *Melipona* core and most abundant ASVs to differentiate between bacteria consistently associated with bees and bacteria found in other environments (S3 Fig). ASVs of *Lactobacillus*, *Bombella* and *Bifidobacterium* groups in *Melipona* are related to those found in other bees, including bumblebee isolates [17]. The *Floricoccus* ASV, although close to environmental isolates, formed a distinct clade together with strains previously isolated from *Melipona* [9]. Similarly, the *Apilactobacillus* ASVs are closely related to *Nicoliella spurrieriana*, a bacterium isolated from *Tetragonula carbonaria*, an Australian stingless bee [18]. These observations point towards two possible stingless bee-associated new clades (Fig. 2C, S3 Fig).

Discussion

The microbiota of *Melipona* differs from that of other eusocial bees, with rare/no occurrence of the symbionts *Snodgrassella* and *Gilliamella*, corroborating previous observations [7–9,19]. The core microbiota is comprised of *Bifidobacterium*, *Lactobacillus*, *Apilactobacillus*, *Floricoccus,* and *Bombella*, as they are present in all bee populations analyzed. In *M. quadrifasciata anthidioides*, the primary microbes

found in the crop, the sugar-rich honey stomach of bees, are *Apilactobacillus* and *Bombella* [18,20]. The ventriculus also has *Apilactobacillus* and *Bombella* as well as several Lactobacillaceae, including the *Lactobacillus* core ASV9 and ASV10. These results are consistent with those of other studies of bee gut microbiota, in which the anterior part of the gut, including the crop and ventriculus, has been found to host environmental and transient microbiota [21].

In other social bees, over 90% of the gut microbiota is found in the hindgut, consisting of ileum and rectum [5]. In Mqa, the rectum is dominated by *Bifidobacterium* and *Lactobacillus*, as observed for other social bees [22,23], but the ileum has a very different composition. The Mqa ileum contains the putative new symbiont close to *Floricoccus* and already isolated from *Melipona* [9] as well as strains of *Bifidobacterium*, Lactobacillaceae (including *Apilactobacillus*), and *Bombella.* In contrast, in honeybees, *Bombella* and *Apilactobacillus* are largely limited to the crop [17,24,25]. Potentially, this distinct ileum community of *Melipona* carries out the same metabolic and defensive functions as the *Snodgrassella-Gilliamella* dominated ileum community of honeybees and bumblebees. Further experimental studies using microbial isolates and bee colonization assays are necessary to explore this issue.

Acknowledgments

We acknowledge the UFV, and financial support from CNPq, CAPES – Finance Code 001 and FAPEMIG (Finance Code APQ – 03029-21) and from US NIH award R35GM131738 to NAM. We thank Anderson Alexandre, Ricardo Marinho Gomes, and Eduardo da Costa Tavares for providing bees.

Data Availability

The 16S rRNA gene amplicon sequencing raw data were deposited in the NCBI BioProject database under the accession number PRJNA1076254.

References

1. Michener CD. The Bees of the World. 2nd ed. The Johns Hopkins University Press; 2007.
2. Hammer TJ, Le E, Martin AN, Moran NA. The gut microbiota of bumblebees. Insectes Sociaux. 2021. pp. 287–301. doi:10.1007/s00040-021-00837-1
3. Kwong WK, Medina LA, Koch H, Sing K, Jia E, Soh Y, et al. Dynamic microbiome evolution in social bees. Sci Adv. 2017;3: 1–17. doi:10.1126/sciadv.1600513

4. Kwong WK, Moran NA. Gut Microbial Communities of Social Bees. Physiol Behav. 2017;176: 139–148. doi:10.1038/nrmicro.2016.43.Gut
5. Martinson VG, Moy J, Moran NA. Establishment of characteristic gut bacteria during development of the honeybee worker. Appl Environ Microbiol. 2012;78: 2830–2840. doi:10.1128/AEM.07810-11
6. Li Y, Leonard SP, Powell JE, Moran A Edited NA, Mcfall-Ngai M. Species divergence in gut-restricted bacteria of social bees. PNAS. 2022;119. doi:10.1073/pnas
7. Cerqueira AES, Hammer TJ, Moran NA, Santana WC, Kasuya MCM, Silva CC da. Extinction of anciently associated gut bacterial symbionts in a clade of stingless bees. ISME J. 2021; 2–5. doi:10.1038/s41396-021-01000-1
8. Haag KL, Caesar L, da Silveira Regueira-Neto M, de Sousa DR, Montenegro Marcelino V, de Queiroz Balbino V, et al. Temporal Changes in Gut Microbiota Composition and Pollen Diet Associated with Colony Weakness of a Stingless Bee. Microb Ecol. 2022. doi:10.1007/s00248-022-02027-3
9. Sarton-Lohéac G, Nunes da Silva CG, Mazel F, Baud G, de Bakker V, Das S, et al. Deep Divergence and Genomic Diversification of Gut Symbionts of Neotropical Stingless Bees. mBio. 2023;14. doi:10.1128/mbio.03538-22
10. Díaz S, de Souza Urbano S, Caesar L, Blochtein B, Sattler A, Zuge V, et al. Report on the microbiota of *Melipona quadrifasciata* affected by a recurrent disease. J Invertebr Pathol. 2017;143: 35–39. doi:10.1016/j.jip.2016.11.012
11. Camargo JMF, Pedro SRM. Meliponini Lepeletier, 1836. de Moelo GAR, Moure JS, Urban D, da Maia E, Dal Molin A, Gonçalves RB, editors. Catalogue of Bees (Hymenoptera, Apoidea) in the Neotropical Region - online version. 2012. Available: http://www.moure.cria.org.br/catalogue
12. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. 2016;13: 581–583. doi:10.1038/nmeth.3869
13. Oksanen J, Simpson GL, Blanchet FG, Kindt R, Legendre P, Minchin PT, et al. vegan: Community Ecology Package. 2022.
14. Wickham H. ggplot2: Elegant Graphics for Data Analysis. ggplot2. Springer New York; 2016. doi:10.1007/978-0-387-98141-3
15. Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. Mol Biol Evol. 2013;30: 772–780. doi:10.1093/molbev/mst010
16. Minh BQ, Schmidt HA, Chernomor O, Schrempf D, Woodhams MD, Von Haeseler A, et al. IQ-TREE 2: New Models and Efficient Methods for Phylogenetic Inference in the Genomic Era. Mol Biol Evol. 2020;37: 1530–1534. doi:10.1093/molbev/msaa015
17. Li L, Praet J, Borremans W, Nunes OC, Manaia CM, Cleenwerck I, et al. *Bombella intestini* gen. nov., sp. nov., an acetic acid bacterium isolated from bumble bee crop. Int J Syst Evol Microbiol. 2015;65: 267–273. doi:10.1099/ijs.0.068049-0
18. Oliphant SA, Watson-Haigh NS, Sumby KM, Gardner J, Groom S, Jiranek V. *Apilactobacillus apisilvae* sp. nov., *Nicolia spurrieriana* gen. nov. sp. nov., *Bombilactobacillus folatiphilus* sp. nov. and *Bombilactobacillus thymidiniphilus* sp. nov., four new lactic acid bacterial isolates from stingless bees *Tetragonula carbonaria* and *Austroplebeia australis*. Int J Syst Evol Microbiol. 2022;72. doi:10.1099/ijsem.0.005588
19. Figueroa LL, Maccaro JJ, Krichilsky E, Yanega D, McFrederick QS, Colleen Cavanaugh EM. Why Did the Bee Eat the Chicken? Symbiont Gain, Loss, and Retention in the Vulture Bee Microbiome. mBio. 2021;12. Available: https://journals.asm.org/journal/mbio

20. Hilgarth M, Redwitz J, Ehrmann MA, Vogel RF, Jakob F. *Bombella favorum* sp. Nov. and *Bombella mellum* sp. nov., two novel species isolated from the honeycombs of *Apis mellifera*. Int J Syst Evol Microbiol. 2021;71: 1–7. doi:10.1099/ijsem.0.004633
21. Romero S, Nastasa A, Chapman A, Kwong WK, Foster LJ. The honeybee gut microbiota: strategies for study and characterization. Insect Mol Biol. 2019;28: 455–472. doi:10.1111/imb.12567
22. Powell JE, Martinson VG, Urban-mead K, Moran A. Routes of Acquisition of the Gut Microbiota of the Honeybee *Apis mellifera*. Appl Environ Microbiol. 2014;80: 7378–7387. doi:10.1128/AEM.01861-14
23. Wu Y, Zheng Y, Wang S, Chen Y, Tao J, Chen Y, et al. Genetic divergence and functional convergence of gut bacteria between the Eastern honeybee *Apis cerana* and the Western honey bee Apis mellifera. J Adv Res. 2022;37: 19–31. doi:10.1016/j.jare.2021.08.002
24. Corby-Harris V, Maes P, Anderson KE. The bacterial communities associated with honeybee (*Apis mellifera*) foragers. PLoS One. 2014;9. doi:10.1371/journal.pone.0095056
25. Anderson KE, Sheehan TH, Mott BM, Maes P, Snyder L, Schwan MR, et al. Microbial ecology of the hive and pollination landscape: Bacterial associates from floral nectar, the alimentary tract and stored food of honeybees (*Apis mellifera*). PLoS One. 2013;8. doi:10.1371/journal.pone.0083125

Supplementary Material

S1 Table. Information of collection, species name and source of the *Melipona* samples analyzed in the present work.

[1*]Bees from the same population collected in different seasons (summer/winter). [2*]Bees from the same population collected in different seasons (summer/winter).

Population Code	Number of Colonies	Sampling Biome	Sampling City	Sampling State	Bee Species	Sub-genus	Source
ME	2	Amazon Forest	Rio Branco	Acre	*Melipona cf. eburnea*	*Michmelia*	New
MCO	4	Amazon Forest	Oriximiná	Pará	*Melipona cf. compressipes*	*Melikerria*	New
MCA1	3	Atlantic Forest	Castelo	Espírito Santo	*Melipona capixaba*	*Michmelia*	New[2*]
MCA2	3	Atlantic Forest	Domingos Martins	Espírito Santo	*Melipona capixaba*	*Michmelia*	New[1*]
MCA3	3	Atlantic Forest	Castelo	Espírito Santo	*Melipona capixaba*	*Michmelia*	New[2*]
MCA4	3	Atlantic Forest	Domingos Martins	Espírito Santo	*Melipona capixaba*	*Michmelia*	New[1*]
MF1	2	Cerrado	Goiânia	Goias	*Melipona cf. fasciculata*	*Michmelia*	New
MF2	2	Amazon Forest	Rio Branco	Acre	*Melipona fasciculata*	*Michmelia*	New
MMA	3	Atlantic Forest	São José	Santa Caratina	*Melipona cf. marginata*	*Eomelipona*	New
MMO	3	Atlantic Forest	São José	Santa Caratina	*Melipona cf. obscuriror*	*Eomelipona*	New
MAL	3	Caatinga	Petrolina	Pernambuco	*Melipona asilvai*	*Eomelipona*	[2]
MAN	3	Atlantic Forest	Santa Tereza	Espírito Santo	*Melipona mandacaia*	*Melipona*	[2]
MAN2	3	Caatinga	Petrolina	Pernambuco	*Melipona asilvai*	*Melipona*	[2]
MIN	3	Amazon Forest	Iranduba	Amazonas	*Melipona interrupta*	*Melikerria*	[2]
MM1	3	Atlantic Forest	Viçosa	Minas Gerais	*Melipona mondury*	*Michmelia*	[2]
MQ1	3	Atlantic Forest	Magé	Rio de Janeiro	*Melipona quadrifasciata*	*Melipona*	[2]
MQ2	3	Atlantic Forest	São Sebastião do Paraíso	Minas Gerais	*Melipona quadrifasciata*	*Melipona*	[2]
MQ3	3	Atlantic Forest	Antônio dos Santos/Caeté	Minas Gerais	*Melipona quadrifasciata*	*Melipona*	[2]
MQ4	3	Atlantic Forest	Viçosa	Minas Gerais	*Melipona quadrifasciata*	*Melipona*	[2]
MQ5	3	Atlantic Forest	Cotia	São Paulo	*Melipona quadrifasciata*	*Melipona*	[2]
MQ6	3	Atlantic Forest	São Paulo	São Paulo	*Melipona quadrifasciata*	*Melipona*	[2]
MQ7	3	Atlantic Forest	Santa Tereza	Espírito Santo	*Melipona quadrifasciata*	*Melipona*	[2]
MQ8	3	Atlantic Forest	Cotia	São Paulo	*Melipona quadrifasciata*	*Melipona*	[2]
MQ9	3	Cerrado	Passos	Minas Gerais	*Melipona quadrifasciata*	*Melipona*	[2]
MRF	3	Atlantic Forest	Santa Tereza	Espírito Santo	*Melipona rufiventris*	*Michmelia*	[2]
MSN	3	Amazon Forest	Iranduba	Amazonas	*Melipona seminigra merrilae*	*Michmelia*	[2]
MSP2	3	Atlantic Forest	São Paulo	São Paulo	*Melipona cf. rufiventris*	*Michmelia*	[2]
MSP1	3	Atlantic Forest	Cotia	São Paulo	*Melipona cf. mondury*	*Michmelia*	[2]
MSP3	3	Atlantic Forest	Cotia	São Paulo	*Melipona cf. scutellaris*	*Michmelia*	[2]
MSP4	3	Atlantic Forest	São Paulo	São Paulo	*Melipona cf. bicolor*	*Eomelipona*	[2]
MSP5	3	Atlantic Forest	Cotia	São Paulo	*Melipona cf. marginata*	*Eomelipona*	[2]
MSP6	3	Amazon Forest	Iranduba	Amazonas	*Melipona cf. rufiventris*	*Michmelia*	[2]
MSU	3	Caatinga	Mossoró	Rio Grande do Norte	*Melipona subnitida*	*Melipona*	[2]

S2 Table. PERMANOVA based on the Bray-Curtis dissimilarity matrix comparing the differences in the microbial community composition between the gut regions of *M. quadrifasciata anthidioides*.

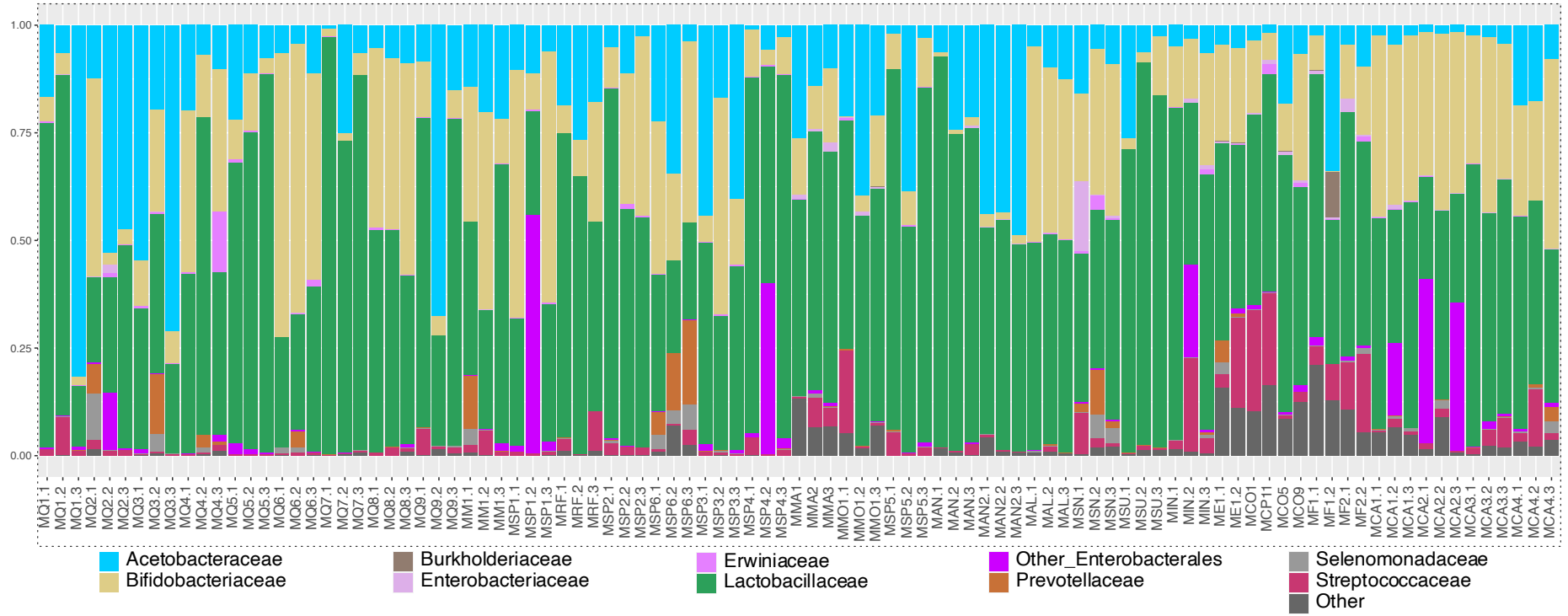
Source	Df	Sum of Squares	R2	F	Pr (>F)
Crop x Ventriculus	1	0.34977	0.16274	3.49880	0.00199
Crop x Ileum	1	0.61699	0.22428	5.20436	0.00199
Crop x Rectum	1	1.47043	0.38016	10.42658	0.00099
Ventriculus x Ileum	1	0.18936	0.07356	1.42922	0.13486
Ventriculus x Rectum	1	1.04267	0.28248	6.69284	0.00099
Ileum x Rectum	1	0.68807	0.18743	3.92140	0.00099
All gut parts	3	2.15244	0.31038	5.25089	0.00099

S3 Table. GenBank sequences used for analysis.

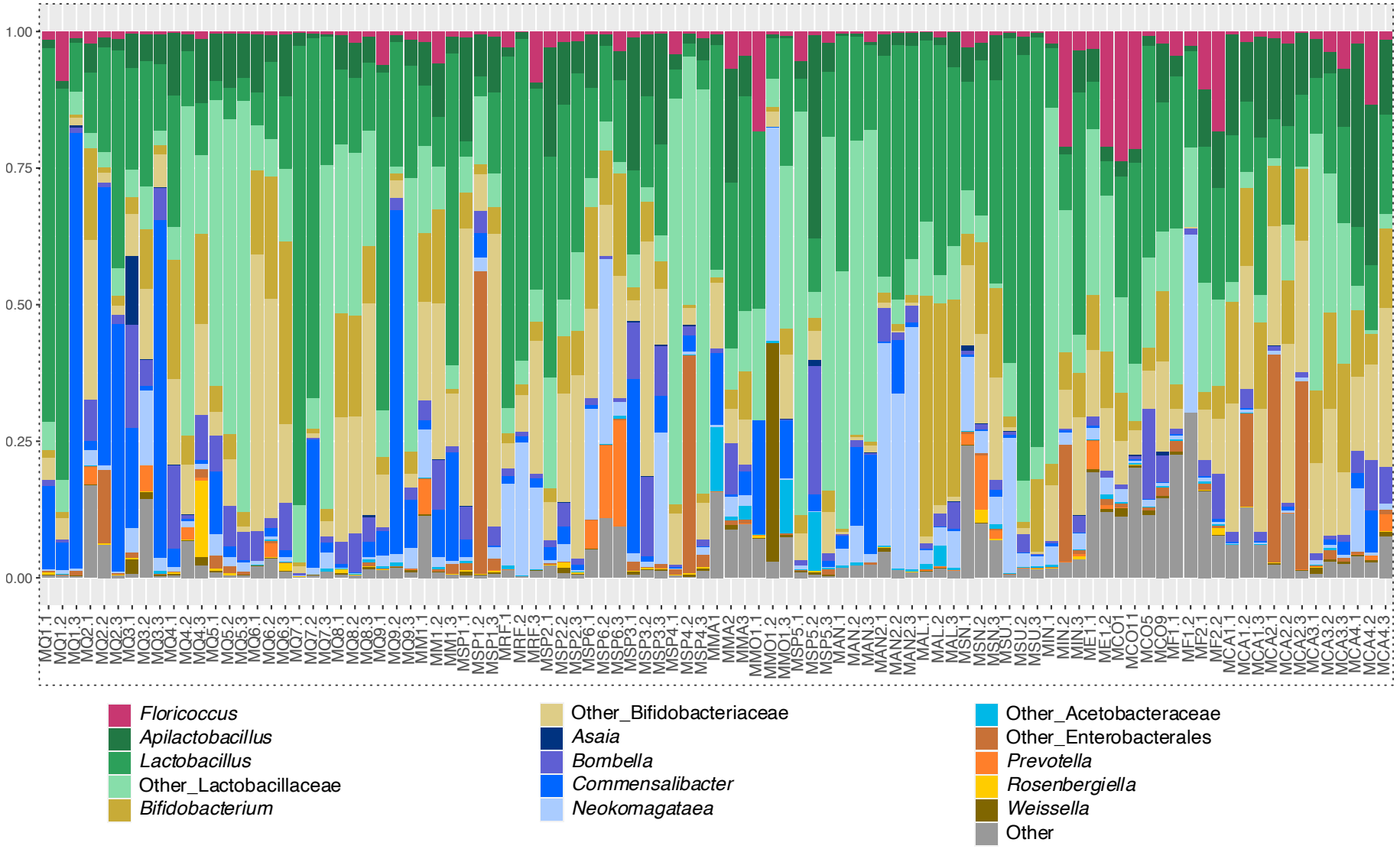
Specie name	Accession	Isolate substrate
	Apilactobacillus tree	
Nicoliella spurrieriana	NR_184611.1	*Tetragonula carbonaria* gut
Nicoliella sp.	OR978307.1	*Lavandula angustifolia* flowers in Spain
Apilactobacillus xinyiensis	NR_181939.1	Honeybee
Apilactobacillus ozensis	NR_113194.1	Mountain flower
Apilactobacillus apisilvae	NR_184610.1	*Austroplebeia australis* whole bee homogenate
Apilactobacillus quenuiae	NR_179014.1	Bee gut (*Augochlorella pomoniella*)
Apilactobacillus timberlakei	NR_179013.1	Flowers of *Abutilon* species
Apilactobacillus bombintestini	NR_174266.1	*Bombus ignitus* gut
Apilactobacillus apinorum	NR_126247.1	*Apis mellifera* honey stomach
Apilactobacillus nanyangensis	NR_179357.1	*Apis mellifera* gut
Apilactobacillus kunkeei	NR_026404.1	Fermented grape juice
Apilactobacillus zhangqiuensis	NR_179374.1	*Apis mellifera* gut
Fructilactobacillus fructivorans	NR_113640.1	Type strain of *Fructilactobacillus fructivorans*
	Lactobacillus tree	
Lactobacillus acetotolerans	NR_117073.1	Chicken crop
Lactobacillus sp. strain Mbhsr5	HM53808.1	Honeybee stomach
Lactobacillus sp. strain MbHmro5	HM534807.1	Honeybee stomach
Lactobacillus sp. strain ESL0731	CP113921.1	*Scaptotrigona polysticta* gut
Lactobacillus sp. strain ESL0700	CP113930.1	*Scaptotrigona polysticta* gut
Lactobacillus sp. strain ESL0677	CP113946.1	*Melipona interrupta* gut
Lactobacillus sp. strain ESL0680	CP113945.1	*Melipona seminigra* gut
Lactobacillus sp. strain MbHmro1	HM534806.1	Honeybee stomach
Lactobacillus sp. strain ESL0785	CP113916.1	*Melipona lateralis* gut
Lactobacillus sp. strain Alhm2to11	HM534787.1	Honeybee stomach
Lactobacillus bombicola	NR_136436.1	Bumblebee gut
Lactobacillus apis	NR_125702.1	Honeybee gut
Lactobacillus panisapium	NR_178998.1	Bee bread
Lactobacillus melliventris	NR_126252.1	*Apis mellifera* honey stomach
Lactobacillus huangpiensis	NR_179358.1	*Apis mellifera* gut
Lactobacillus laiwuensis	NR_179376.1	*Apis mellifera* gut
Lactobacillus kimbladii	NR_126250.1	*Apis mellifera* honey stomach
Amylolactobacillus amylopilus	NR_113816.1	Type strain of *Amylolactobacillus amylopilus*
	Streptococcacea tree	
Streptococcaceae bacterium	CP113940.1	*Melipona seminigra* gut
Streptococcaceae strain MM128	Non-published	*Melipona mondury* gut
Floricoccus tropicus	NR_159226.1	Flowers of durian tree and *Hibiscus*
Floricoccus penangensis	NR_159225.1	Flowers of durian tree and *Hibiscus*
Streptococcaceae bacterium	CP113924.1	*Scaptotrigona polystica* gut
Lactococcus piscium	NR_043739.1	Broiler carcasses
Lactococcus plantarum	NR_044358.1	Activated sludge foam
Lactococcus lactis	NR_113958.1	Type strain *Lactococcus lactis subsp. Hordniae*
Lactococcus taiwanensis	NR_114327.1	Pobuzihi, traditional fermented food in Taiwan
Streptococcus agalactiae	NR_113262.1	Type strain of Streptococcus agalactiae
Streptococcus tangierensis	NR_134818.1	Raw camel milk
Streptococcus cameli	NR_134817.1	Raw camel milk
Streptococcus thoraltensis	NR_026368.1	Genital tract of sows
Streptococcus hyovaginalis	NR_044912.1	Genital tract of sows
Lactiplantibacillus plantarum	NR_113338.1	Type strain of *Lactiplantibacillus plantarum*

Bifidobacteriaceae tree		
Bifidobacterium minimum	NR_044692.2	Sewage
Bifidobacterium indicum	NR_043439.1	Hindgut of honeybee
Bifidobacterium callitrichos	NR_113172.1	Faeces of common marmoset (*Callithrix jacchus*)
Bifidobacterium rousetti	NR_164634.1	Faeces of Egyptian fruit bat
Bifidobacterium asteroids	NR_044154.1	*Apis mellifera* honey stomach
Bifidobacterium bombi	NR_104872.1	Digestive tract of *Bombus lucorum*
Bifidobacterium bohemicum	NR_108439.1	Bumblebee digestive tract
Bifidobacterium sp. ESL0798	CP113914.1	*Scaptotrigona polysticta* gut
Bifidobacterium sp. ESL0728	CP113925.1	*Melipona fuliginosa* gut
Bifidobacterium sp. ESL0704	CP113929.1	*Scaptotrigona* sp. gut
Bifidobacterium sp. ESL0790	CP113915.1	*Melipona fuliginosa* gut
Bifidobacterium sp. ESL0690	CP113939.1	*Melipona lateralis* gut
Bifidobacterium sp. ESL0682	CP113942.1	*Melipona fuliginosa* gut
Bifidobacterium commune	NR_136422.1	Bumblebee gut
Bifidobacteium sp. ESL0769	CP113918.1	*Melipona fuliginosa* gut
Bifidobacterium sp. ESL0764	CP113919.1	*Scaptotrigona polysticta* gut
Bifidobacterium sp. ESL0732	CP113920.1	*Scaptotrigona polysticta* gut
Bombiscardovia coagulans	NR_116179.1	Bumblebee digestive tract

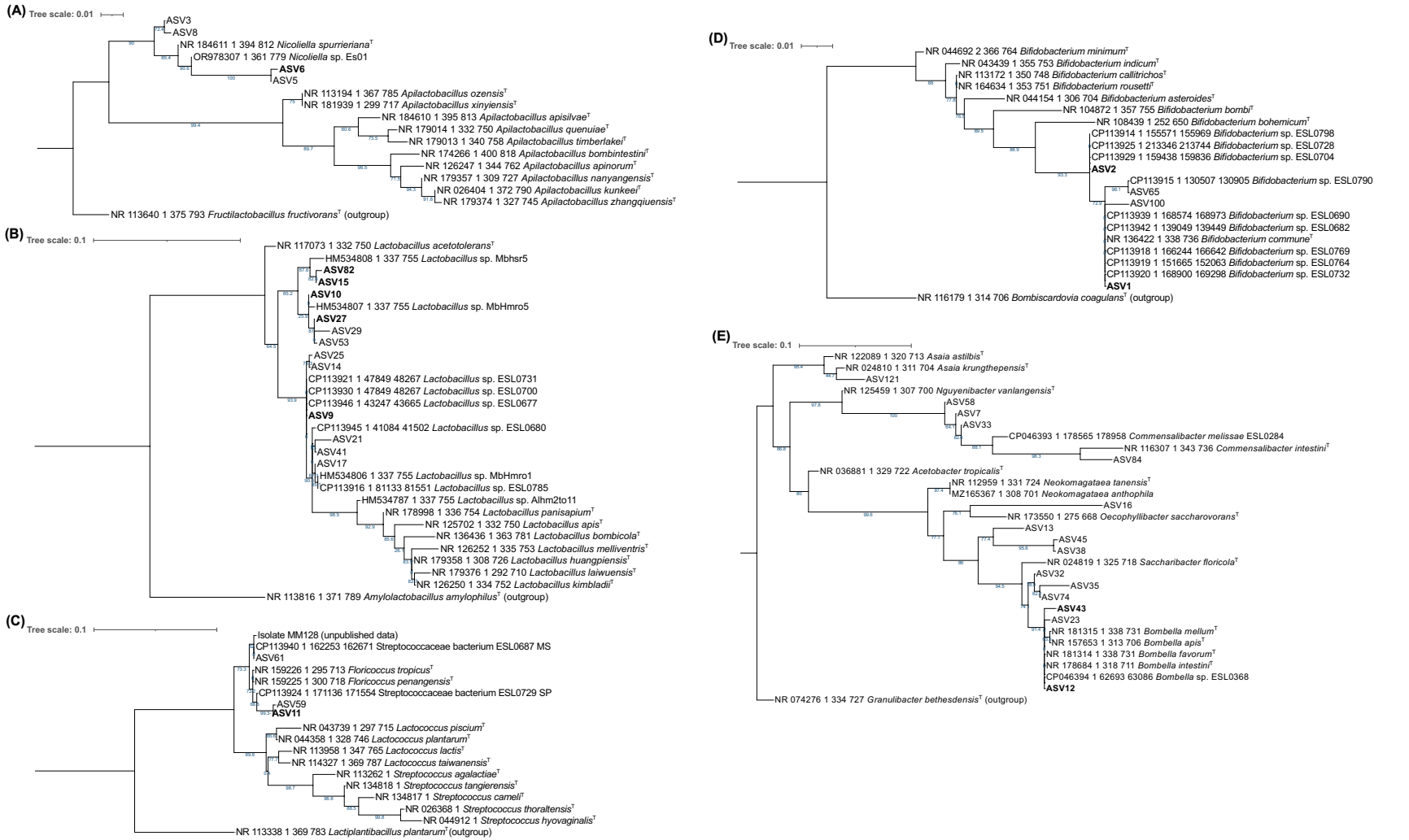
Acetobacteraceae tree		
Asaia atilbis	NR_122089.1	Japanese flowers
Asaia krungthepenses	NR_024810.1	*Heliconia* sp. flower
Nguyenibacter vanlangensis	NR_125459.1	Rhizosphere of Asian rice
Commensalibacter melissae	CP046393.1	Honeybee gut
Commensalibacter intestini	NR_116307.1	*Drosophila melanogaster* gut
Acetobacter tropicalis	NR_036881.1	Type strain of *Acetobacter tropicalis*
Neokomagataea tanensis	NR_112959.1	Candle bush flower
Neokomagataea anthophila	MZ165367.1	Flower
Oecophyllibacter saccharovorans	NR_173550.1	Weaver ant *Oecophylla smaragdina*
Saccharibacter floricola	NR_024819.1	Pollen
Bombella mellum	NR_181315.1	Honeycomb of *Apis mellifera*
Bombella apis	NR_157653.1	Midgut of honeybee
Bombella favorum	NR_181314.1	Honeycomb of *Apis mellifera*
Bombella intestini	NR_178684.1	Bumblebee crop
Bombella sp. strain ESL0368	CP046394.1	Honeybee gut
Granulibacter bethesdensis	NR_074276.1	Type strain of *Granulibacter bethesdensis*



S1 Figure. Most abundant families in *Melipona* spp. gut microbiota. Each sample is related to one pool of bees per box per site of study. ASVs are ordered and colored at the family level, with low abundant ASVs grouped as 'Other'.



S2 Figure. Most abundant genera in *Melipona* spp. gut microbiota. Each sample is related to one pool of bee per box per site of study. ASVs are ordered and colored at the genus level, with low abundant ASVs grouped as 'Other'.



S3 Figure. Phylogenetic trees of the most abundant ASVs (including the 11 core ASVs) found in *Melipona* bee populations. Bootstrap values are shown in blue letters. The 11 core ASVs are written in bold characters. ^{T} Type strain. Trees are shown for the most abundant and core ASVs of A) *Apilactobacillus*, B) *Lactobacillus*, C) Streptococcaceae, D) Bifidobacteriaceae, and E) Acetobacteraceae.

CAPÍTULO 2

Seasonal and landscape influences on the gut microbiota of the endangered *Melipona capixaba* bees

(Capítulo formatado de acordo com as normas da revista PLOS ONE)

Abstract

The relationship between insects and microorganisms is fundamental to the evolutionary success of both groups. Bees, in particular, rely on their specialized gut microbiota for their overall well-being. Among eusocial bees, *Melipona* bees stand out due to their unique core microbiota that sets them apart from other species. However, very little is known about *M. capixaba*, an endemic species found exclusively in Espírito Santo state, Southeastern Brazil, that is currently endangered of extinction. This research seeks to address the gap in knowledge by analyzing the gut microbiota of *M. capixaba* collected from different landscapes at summer and winter seasons. The total DNA was extracted from a pool bees per colony and had the 16S rRNA V3-V4 regions sequenced. The study revealed that the microbial community of these bees is influenced by both the landscape and the season. Bees collected during the summer of 2023 had a gut microbiota that differed significantly from other time points. Furthermore, bees from urban areas had a distinct microbial community compared to bees from farmland, with variations observed between summer and winter, too. These results pointed some important bacterial groups in *M. capixaba* microbiota, such as members of Bifidobacteriaceae, Acetobacteraceae, Streptococcaceae and Enterobacterales. The results showed that, in general, seasonal and landscape influences in *M. capixaba* gut microbiota.

Introduction

The interdependence between insects and microorganisms is crucial for the diversification and evolutionary success of insects [1]. A wide range of microorganisms inhabit the gut of these animals and bring benefits ranging from increased oviposition, longevity, decreased larval period, and increased resilience to environmental

disturbances or changes in host behavior [2–4]. Social bees have a dense and specific gut microbiota composed of core members present in multiple bee species, as well as environmental-transient bacteria [5]. These microorganisms are essential in maintaining the bee's wellness, being involved, for example, in the upregulation of antimicrobial peptide production in the bee [6], in the pollen digestion [7], and detoxification of the bee against harmful chemical compounds [8]. Bees acquire their microbiome through social interactions with their colony members and the surrounding environment and diet [5,9].

Among the corbiculate bees, *Melipona* Iliger [10] is a genus belonging to the Meliponini, which includes the biggest diversity of species occurring in the Neotropical region. *Melipona* (*Michmelia*) *capixaba* Moure and Camargo 1994 [11], popularly known as uruçu-capixaba, is endemic to the Brazilian Atlantic Forest, restricted to areas in mountainous regions of Espirito Santo, Brazil [12]. Together with other Meliponini, this bee is included in the List of Species of Brazilian Fauna Threatened with Extinction [13] because of the fragmentation of its natural habitat. There is limited information available on the ecology and biology of *M. capixaba* [14], and so far, no studies have been conducted on its gut microbial composition.

Melipona bees have a unique core microbiota that differs from other eusocial bees, comprising *Apilactobacillus, Lactobacillus, Bifidobacterium, Floricoccus*, and *Bombella* [15–17]. Most of the research on the gut microbiota of social bees relies on studies on honeybees and bumblebees. It is known that the bee gut microbiota is influenced by host age, gut morphology, caste [18], and the environment [9]. Disturbance in the bee gut microbiota can disrupt their beneficial influence on the host. Environmental toxicants, for instance, can indirectly impact the bumblebee's health by disrupting their gut microbiota [19]. Similarly, other environmental stressors can also lead to dysbiosis. The use of agrochemicals and antibiotics affects the gut microbiota of honeybees and makes them susceptible to the infection of opportunistic pathogens [20, 21].

Environmental changes also impact the bee gut microbial community. Throughout the foraging season, the microbiota of honeybees undergoes significant changes, while it remains stable during winter [22]. In *M. quadrifasciata*, a bee that overgoes a colony collapse syndrome usually at the end of summer, it was reported a temporal variation in the bee gut bacterial community two months before the syndrome,

with a change in their forage behavior [16]. Australian stingless bees presented a shift in their gut microbiota following colony relocation, which persisted over long periods of time after returning the hives to the original site [23].

The *M. capixaba* bee plays a crucial role in pollination within its natural habitat and holds social significance for local beekeepers. However, there is limited information available about the microbiota of *M. capixaba*. Therefore, this study seeks to investigate how the landscape and seasonal variations impact the gut microbiota of *M. capixaba* bees, which are native to Espirito Santo state, Brazil. Our hypothesis suggests that the gut microbiota of these bees is influenced by landscape conditions and weather patterns.

Methodology

Sample collection and season conditions

To assess the influence of landscape on the microbiota associated with *M. capixaba* bees in areas of the Atlantic Forest biome in Espírito Santo, Brazil, forager bees were collected under four environments: (1) natural areas, where there was some agricultural influence in the bee's flight path, (2) an organic farm that produced organic coffee, (3) farmland areas (farm), which were characterized as small-scale farming, and (4) urban areas. (Fig. 1). The region has a Cwb climate according to Koppen's classification, with a dry winter and a temperate summer [24]. Therefore, the collection was carried out at the end of the rainy and hot season (summer) and at the end of the cold and dry season (winter): summer/2022: natural1, natural2, farm1, farm2, urban1, urban2 and urban3; winter/2022: natural1, natural2, farm1, farm2, farm3, organic farm, urban1, urban2 and urban3; summer/2023: natural3, farm3 and organic farm. Based on the availability of the beehives, we collected samples at the end of summer and winter in 2022 and summer in 2023. In the summer of 2023, we only collected samples from the natural3, farm3, and organic farms because they were not available during the summer of 2022. We collected 15 adult bees from the entrance of three colonies per collection site and placed them in sterile 50 mL tubes (Falcon type) containing 95% ethanol. The samples were stored at -20ºC until further use.

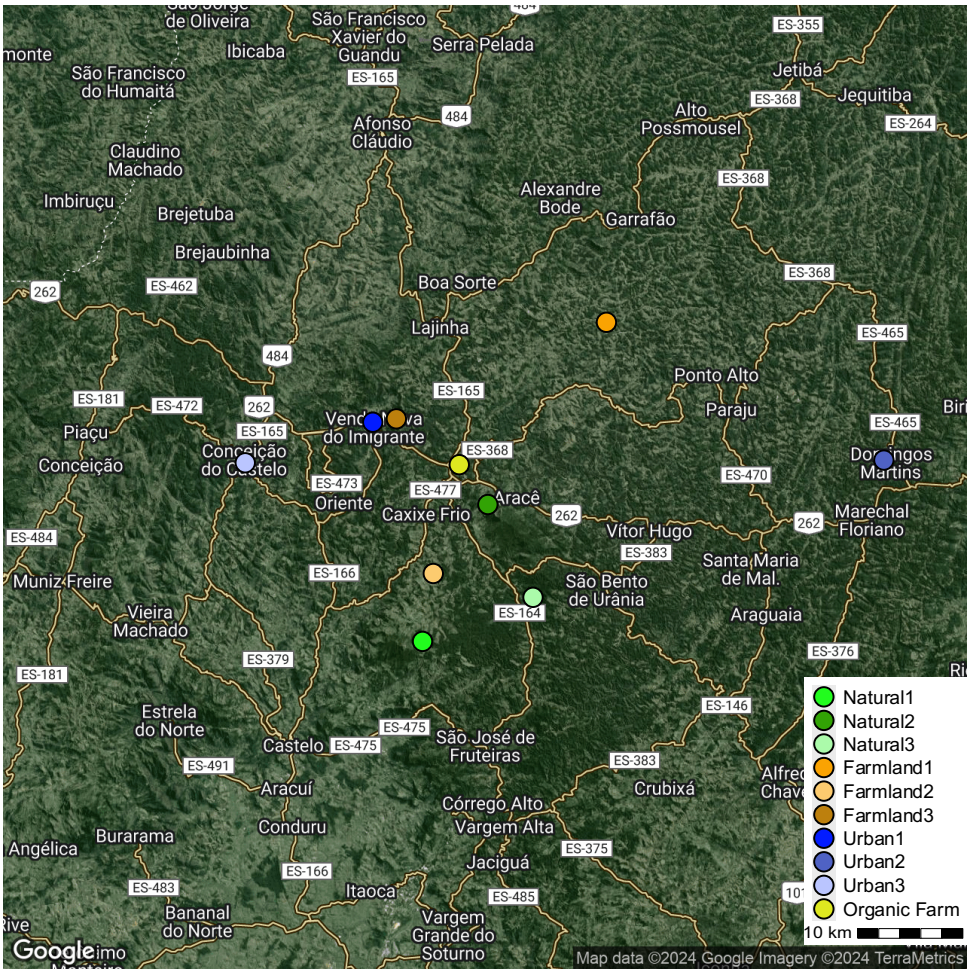


Figure 1. **Collection sites of *Melipona capixaba* samples in Espirito Santo state, Brazil.** Source: Google Maps.

To better understand the weather patterns leading up to our sample collection, the four months preceding the sample collection were categorized into three groups: the summer of 2022 (November 2021 to February 2022), the winter of 2022 (May 2022 to August 2022), and the summer of 2023 (November 2022 to February 2023). We obtained the data from the automatic meteorological station number A633 situated in Venda Nova do Imigrante, Espirito Santo state, Brazil, as provided by the Brazilian National Institute of Meteorology. Samples collected for summer 2022 were obtained in early March 2022, while samples collected for winter 2022 were obtained in early September 2022. Lastly, samples collected for summer 2023 were obtained in mid-February 2023.

Agrochemical analysis

To assess the potential impact of agrochemicals on the gut microbiota of those bees, we gathered honey samples from each collection site in May of 2023. At each location, we examined a blend of honey collected from the accessible beehives to detect any traces of pesticides. These samples were then transported to the Laboratory of Pesticide Residues Analysis at the Federal University of Santa Maria in Brazil. To detect the presence of pesticide residues was employed ultraperformance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) utilizing the cutting-edge Waters Xevo TQ MS with Acquity UHPLC system, following the QuEChERs methodology [25].

DNA extraction, 16S rRNA gene sequencing, and data processing

Bees were superficially disinfested in a 1% NaClO (sodium hypochlorite) solution, washed with sterile distilled water, and dissected using sterile forceps under a stereoscopic microscope. For the DNA extraction, the samples comprised a pool of five bee guts per colony assessed. The total genomic DNA extracted with the NucleoSpin Soil Kit (Macherey-Nagel), preceded by a proteinase K treatment for 2 hours at 56ºC [15]. The DNA samples were submitted for 250 bp paired-end amplicon sequencing at Novogene Corporation Inc (Sacramento, CA, USA) using an Illumina NovaSeq 6000 System. The primer pair 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACNNGGGTATCTAAT) targeted the 16S rRNA V3-V4 regions.

The data was processed using the DADA2 package (version 1.28) [26] in R 4.3.1, following the pipeline available at https://benjjneb.github.io/dada2/tutorial.html. First, Cutadapt [27] was used to remove adapters. Then, the DADA2 pipeline was followed to filter, trim, de-replicate, denoise, and merge the paired-end sequences to construct the amplicon sequence variant (ASV) table. The taxonomy of the ASVs was assigned using a trained SILVA database (version 138.1 from November 2020) for bacteria [28].

The data analysis was conducted using three R packages: "mctoolsr" version 0.1.1.9, available at https://github.com/leffj/mctoolsr, "vegan" version 2.6-4 [29], and "ggplot2" version 3.4.2 [30]. The first step was to remove mitochondria, chloroplasts, and low-abundance ASVs (those with less than 120 total reads across all samples)

from the dataset. The dataset was then rarefied to ensure an equal number of sequences per database. Alpha diversity was assessed using the Shannon and richness metrics. The relative abundances (beta diversity) were used to create a dissimilarity matrix based on the Bray-Curtis distance. The "Adonis" function was employed to perform the permutational multivariate analysis of variance (PERMANOVA) and Kruskal-Wallis test.

Results

Influence of the landscape in the bee gut microbiota

The gut microbiota of *M. capixaba* bees is influenced by the landscapes they inhabit. To test this hypothesis, we analyzed 57 samples, totaling 10,244,370 16S rRNA sequences. After quality filtering, the number of sequences per sample ranged from 57,610 to 152,710 reads. These sequences clustered into a total of 989 different ASVs. We then filtered out low abundance taxa (>120), resulting in a summary of 152 different ASVs. We identified ASVs at the genus level, but for those identified only at the family or order level, we classified them as "Other" along with the family or order name.

The results indicated that alpha diversity, as measured by the Shannon diversity index, remained consistent across different landscapes (Figure 2). However, the Bray-Curtis dissimilarity matrix, assessed by pairwise PERMANOVA, showed that bees in urban areas have a different microbial community compared to bees from other locations. Conversely, bees from farmland, organic farm and natural areas exhibited a similar microbiota (Table 1).

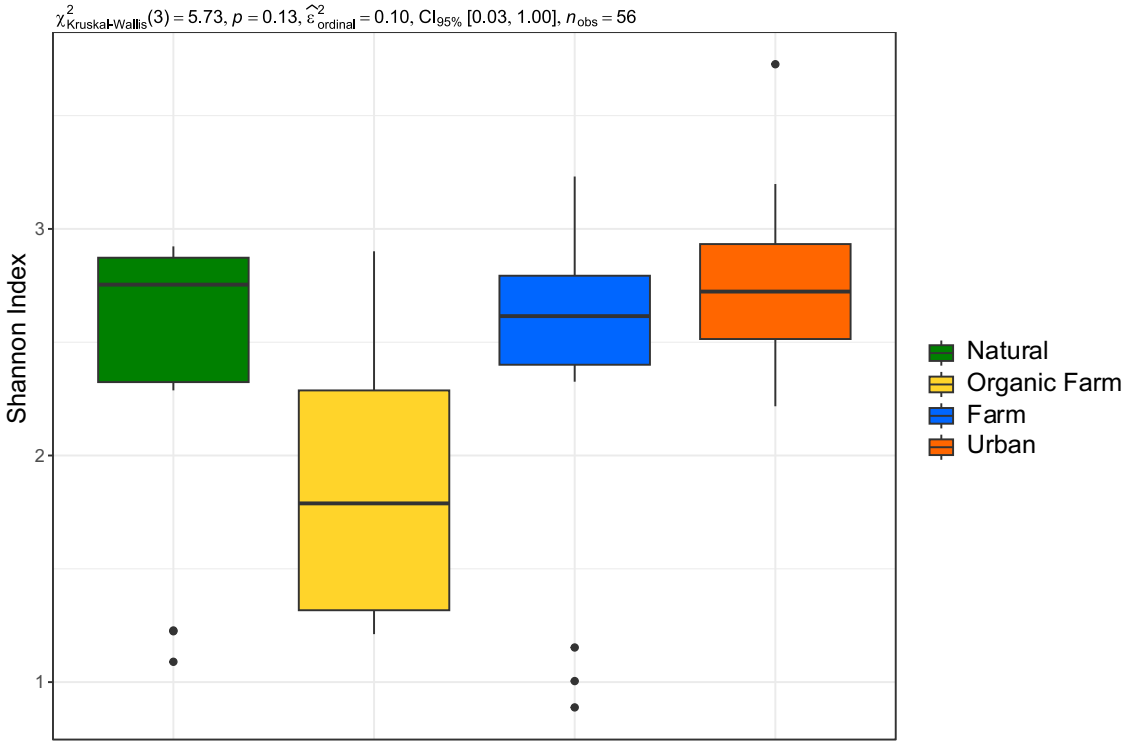


Figure 2. Bacterial alpha diversity of the gut microbiota of *M. capixaba* collected in different landscapes. The alpha diversity was expressed using the Shannon index. A Kruskall Wallis test ($p < 0.05$) was conducted, followed by a post-hoc pairwise Dunn test to compare each landscape. No statistical difference was observed.

Table 1. Pairwise comparison of the gut bacterial community of *M. capixaba* in different landscapes and seasons. Analysis was performed using PERMANOVA tests on the Bray-Curtis dissimilarity matrix ($p < 0.05$).

Landscape comparison	**Df**	**SumOfSqs**	**F.Model**	**R^2**	**p-value**
Natural vs urban	1	0.3444	3.0269	0.0916	0.001*
Natural vs farmland	1	0.1289	1.0451	0.0326	0.347
Urban vs farmland	1	0.3068	2.6352	0.7040	0.008*
Urban vs organic farm	1	0.6921	6.5140	0.2368	0.001*
Farmland vs organic farm	1	0.2867	2.3864	0.0979	0.044

Season comparison	**Df**	**SumOfSqs**	**F.Model**	**R^2**	**p-value**
Summer/22 vs winter	1	0.2114	2.3430	0.0495	0.007*
Summer/22 vs summer/23	1	2.1788	28.4963	0.5043	0.001*
Winter vs summer/23	1	2.3089	33.4217	0.5031	0.001*

Season vs landscape	Df	SumOfSqs	F.Model	R^2	p-value
Natural	1	0.1209	1.8278	0.1545	0.067
Organic Farm	1	0.4241	18.3813	0.8212	0.1
Farmland	1	0.1274	1.5398	0.1059	0.076
Urban	1	0.2277	2.2974	0.1328	0.028*

*Difference at 5% of significance level.

The composition analysis of samples comprised bacteria from core genera such as *Bifidobacterium, Lactobacillus, Bombella*, and *Floricoccus* (Fig. 3), converging with the findings in the first chapter of the present thesis. Additionally, samples from natural2, farm1, farm2, and urban areas contain Enterobacterales, which is less prevalent in other areas and not found in most of the samples from the organic farm. Interestingly, urban areas have a higher mean relative abundance of Enterobacterales, and a lower abundance of *Bifidobacterium* than other areas.

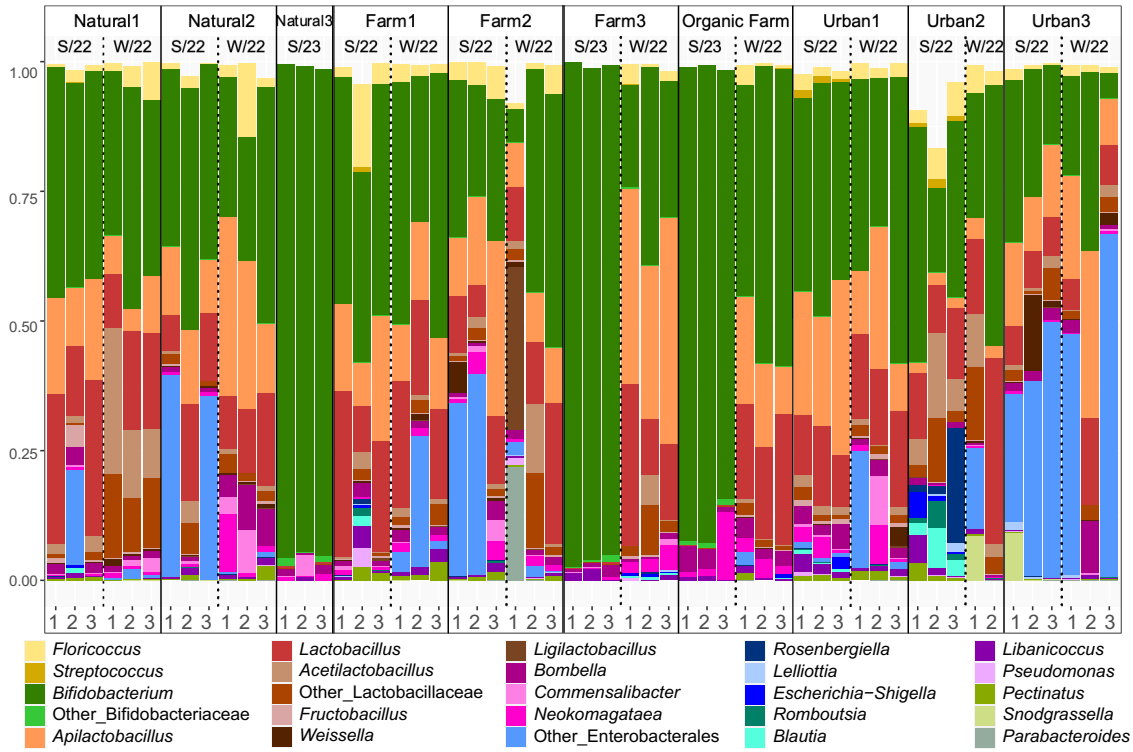


Figure 3. Mean relative abundance (>0.001) of bacterial genera present in the gut of *M. capixaba* collected in different landscapes and seasons. S/22: summer of 2022; S/23: summer of 2023; W/22: winter of 2022.

At the ASV level (Fig. 4), it was noteworthy that the samples share a group of common ASVs for all landscapes, with ASV1 and ASV2 (both *Bifidobacterium*) being the most abundant in all regions. Natural areas possess a similar abundance of ASV14 related to *Floricoccus*, while urban and organic farm areas exhibit a lower abundance of the same ASV. ASV4 and ASV7, both Enterobacterales, had greater abundance in urban areas. Overall, organic farm tends to have a lower bacterial community diversity, reflected in their lower diversity. The remaining primary ASVs related to *Apilactobacillus* and *Lactobacillus* had a comparable abundance within the different landscapes.

In the analysis of pesticides, no residues were found in any of the honey samples. The report for one of the samples is included in the supplementary material for this work.

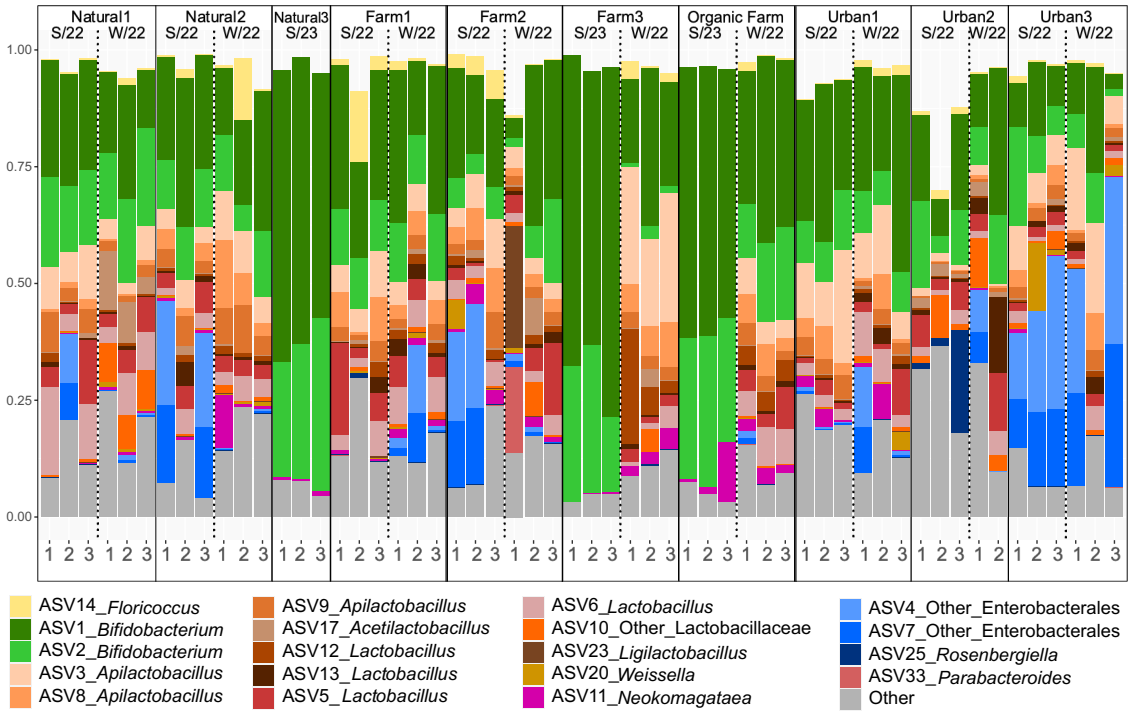


Figure 4. **Mean relative abundance (>0.001) of the 20 most abundant ASVs in *M. capixaba* collected in different landscapes and seasons.** S/22: summer of 2022; S/23: summer of 2023; W/22: winter of 2022.

In total, the bees collected from different landscapes shared 110 ASVs out of 152 (as shown in Fig. 5). Among the ASVs, 28 ASVs were common among the bees collected from natural, farmland, and urban environments. The ASV140

(*Acetilactobacillus*) was specific to the natural environments, while the ASV114 (*Parabacteroides*) was specific to farmlands, and the ASV173 (*Blautia*) was specific to urban areas. However, no specific ASVs were found in the organic farm area.

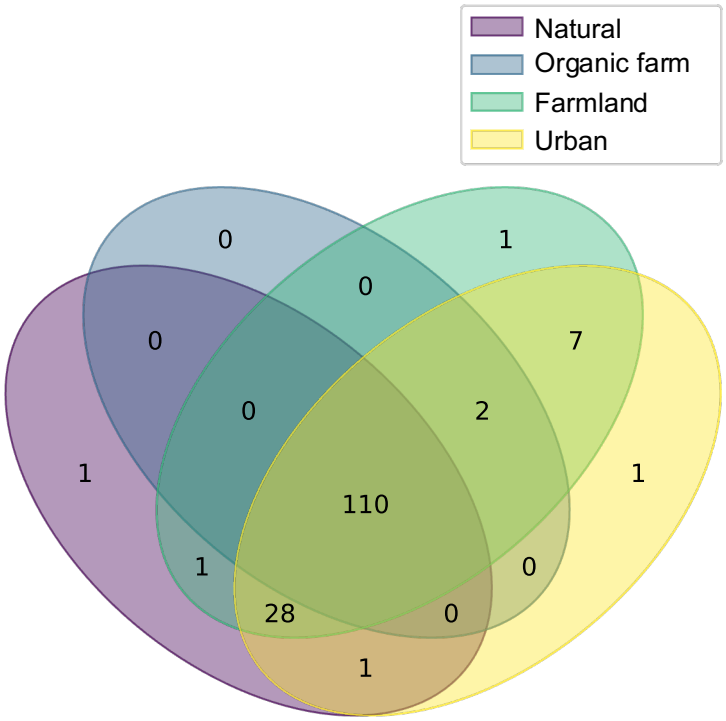


Figure 5. Venn diagram of ASVs present in the bee gut microbiota of *M. capixaba* across environments. Number of shared and unique ASVs across different landscapes: 110 ASVs are shared between all the landscapes; 28 ASVs are shared between farmland, urban and natural; 7 ASVs are shared between farmland and urban; 2 ASVs are shared between farmland, organic farm and urban; 1 ASV is shared between natural and farmland; 1 ASV is shared between natural and urban. Each of the natural, farmland and urban landscapes has one unique ASV.

The gut microbial community in M. capixaba varies seasonally

Between November 2021 and February 2022, the summer temperatures saw a range of 19.3 ºC to 21.8 ºC. The rainfall levels during this period ranged from 208.8 to 224.4 mm, with a significant increase to 478.4 mm in February 2022. The subsequent winter of 2022 experienced slightly lower temperatures ranging from 15.6 to 17.9 ºC through May, June, July, and August 2022. The rainfall levels were comparatively low during these months, ranging from 14.6 to 1.8 mm. Moving on to the summer of 2023,

the temperatures ranged from 19.6 to 21.8 ºC between November 2022 and February 2023. The rainfall levels varied between 167.6 mm in November, 359.8 mm in January, and 46.6 mm in February.

There was a significant difference in the microbial composition of the samples collected in the summer of 2023 compared to those from the previous summer and winter (Figure 6, Table 1). The gut microbiota of bees from winter showed greater similarity independently of landscape. Additionally, when comparing each landscape in each season, it was observed that the microbiota of bees in urban areas differed significantly between summer and winter seasons, although this pattern was not observed in other landscapes (Table 1).

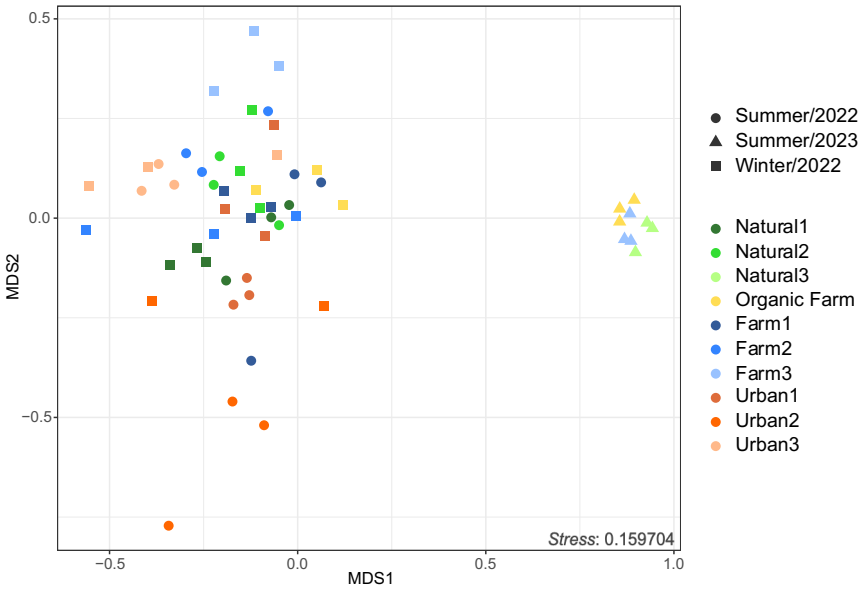


Figure 6. **NMDS based on ASV relative abundance (Bray-Curtis dissimilarity) in *M. capixaba* sampled in different landscapes and seasons.**

The gut microbiota of bees collected in the summer of 2023 was dominated by *Bifidobacterium* ASV1 and ASV2, regardless of the collection site; we believe that this was the reason for lower Alpha diversity observed in the summer of 2023 samples (Fig 4 and Fig. 7). During the winter of 2022, organic farm and farm3 presented a higher alpha diversity of ASVs than in the summer of 2023 (Fig. 7). For natural areas samples collected in the summer and winter of 2022, a slightly higher relative abundance of *Floricoccus* (ASV14) was observed in winter, whereas for farmland areas, this ASV was more abundant in summer. The Enterobacterales ASV4 and ASV7 were more abundant in natural2 and farm2 during the summer and in both seasons for urban

area3. Urban area2 presented a higher abundance of other ASVs in the summer, which could be related to environmental ASVs.

The gut bacterial composition of bees collected in the same environment, but different seasons showed a significant difference in alpha diversity, as represented by the Shannon index (Fig. 7).

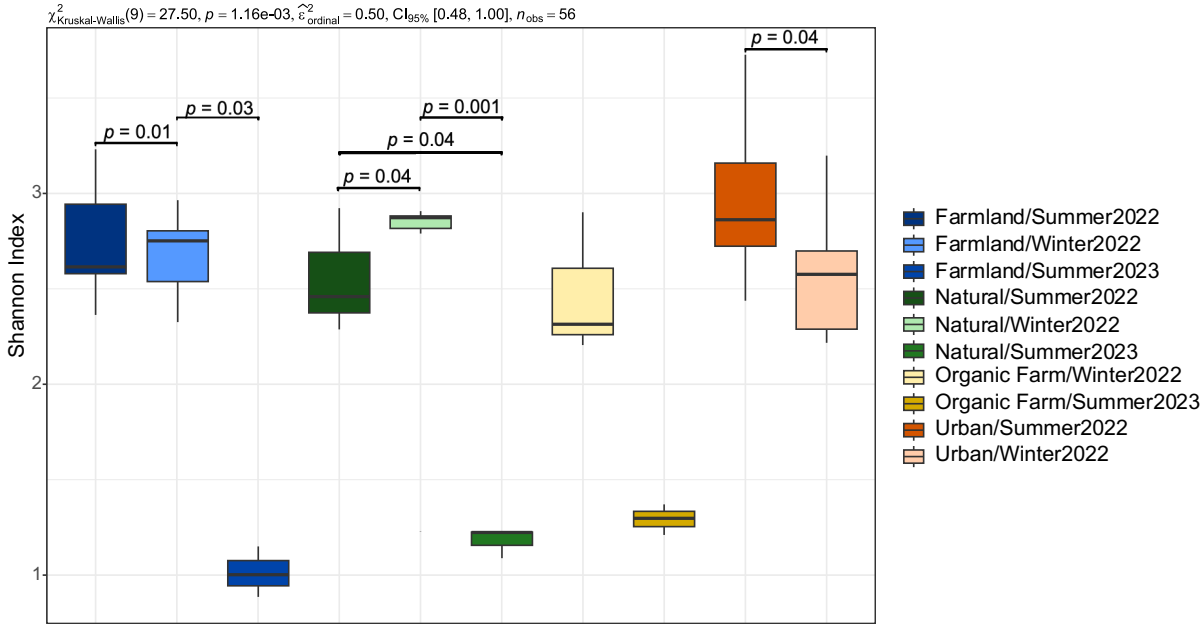


Figure 7. Bacterial alpha diversity of the gut microbiota of *M. capixaba* collected in different landscapes and seasons. The alpha diversity was expressed using the Shannon index**.** A Kruskal-Wallis test ($p < 0.05$) was conducted, followed by a post-hoc pairwise Dunn test to compare each landscape in the time point collections, showing only the significant results.

Discussion

Social bees, including *Melipona* spp., have a core microbiota with low diversity that typically contains *Bifidobacterium*, *Lactobacillus*, *Apilactobacillus*, *Bombella*, and *Floricoccus*. There are also environmental-associated bacteria present in their microbiome, as previous research has shown [2,8,9].

Our study found that the relative abundance of certain members of the core microbiota in *Melipona* forager bees varies depending on the landscape (natural, urban, farmland, or organic farm areas). Additionally, there was no difference in microbial community diversity (as measured by the Shannon diversity index) between landscapes.

Environmental factors have a significant impact on bee gut microbiota. Stingless bees were susceptible to microbial compositional changes that could be triggered by a variety of factors, such as food resources [16], physiological status [31], colony origin [11], and climate at different geographic locations [12,13]. It's also known that the bee gut microbiota can be impacted using agrochemicals [32–36], which can indirectly affect bees through sources like pollen, nectar, and water [37]. However, our study found no detectable pesticide residues in the honey produced by *M. capixaba*, possibly due to the timing of honey collection or the sensitivity of the testing method. The region where the study took place is renowned for eco-tourism and small-scale family farms, which tend to use fewer or no agrochemicals, resulting in more responsible landscape management.

There is a noticeable composition difference in the bee gut microbiota in the summer of 2023 compared to other seasons. During this time, *Bifidobacterium* dominated in the samples, which was consistent with the low alpha diversity level observed during this period. Unfortunately, we were unable to collect bees from other sites to compare with those collected during the summer of 2022. The weather conditions during this time, with intense rainfalls in December 2022 and January 2023, may have affected bee pollination. Prolonged periods of heavy rain could disturb floral resource availability [38], potentially dilute nectar, and degrade pollen [39], which are crucial factors in bee diet. These factors, combined with the dominance of *Bifidobacterium* could inhibit the growth of other microorganisms in the community by producing antimicrobial substances [40–42], contribute to the low bacterial diversity observed during the summer of 2023.

Regardless of season, the bacterial community was, in general, influenced by the seasonally. The samples collected at the end of the winter of 2022 presented a higher similarity than those from the summer of 2022. Winter bees were, in general, confined to the hive, feeding mainly on stored honey [43]. Colder and drier days change the bees' behavior, which reflects their outside activity. For stingless bees, colder days reduce activity during the coldest hours of the day, but dry days can force the bees to go out more often to get water [44]. The foraging behavior is also impacted by the availability of resources during that period [45]. In that way, the microbiota of the bees in the winter tended to be more similar as they had less contact with environmental and transient microorganisms due to the shorter foraging time.

Notably, bees collected from urban areas had a gut microbiota significantly different from bees collected in other landscapes. The higher mean relative abundance of *Enterobacterales* bacteria in these samples, particularly in the urban3 site, indicates stress on bee health, as these bacteria are commonly associated with gut dysbiosis and unhealthy colonies [46–48]. Enterobacteriaceae are typically found in the hive microbiome of different species of stingless bees [49], and *Melipona* bees collect plant material, mud, and animal feces to build their nests [50].

The impact of seasonal changes on the microbial community of bees was more pronounced in urban areas. Having access to a diverse range of flowering plants is crucial to improve the resilience of gut bacteria in bees against different climatic variables. When floral resources are limited or disrupted, the microbiome of bees can suffer from a depletion of environmental microorganisms [23,51]. Differences in floral availability can significantly impact the variations between urban and non-urban areas [52], with this effect being more pronounced in different seasons in urban areas, which can relate to the results observed in the present work. *Melipona* bees typically have generalist feeding habits and rely on rich floral sources for pollen and nectar [53]. Urban areas usually have fewer floral resources, particularly during winter, significantly affecting the bee gut bacterial community. Urbanization [52] and dietary changes [54,55] have been found to affect the gut bacterial community in bees. Studies have shown that green habitats can reduce parasite infection in *Bombus pascuorum* [56]. Bees from highly urbanized areas exhibit a greater diversity of bacteria, while winter bees are more sensitive to dietary changes [57]. In urban *Ceratina calcarata* bees and their pollen provisions, there is a higher level of microbial diversity. However, the microbiota of these bees has lower relative abundances of key symbionts and higher levels of pathogens [58]. Climate change has a significant impact on the gut microbiota of bees, affecting the establishment of symbiotic relationships with microorganisms [59]. The loss of natural habitats due to urbanization and agriculture, along with climate change, is a major contributor to the extinction of species over the next century [60]. The seasonal changes in the gut microbiota of stingless bees that this study documents were particularly concerning, given the existing challenges that these insects face as a result of human activities both now and in the future.

Conclusion

M. capixaba microbiota comprises the core members previously described for other *Melipona* bees. This core microbiota is consistent along all the samples in this work. However, we observe that the microbial community in the bee gut is influenced by the seasonally and the landscape. In particular, we verify that the microbiota of bees in urban areas is significantly different from those in other landscapes. Moreover, we observed that the seasonally play a key role in shaping the microbial community of bees. During the summer of 2023, we detected a significant difference in the microbial community, with *Bifidobacterium* being the dominant genera. We also noted that bees in urban areas had different microbiomes in summer and winter, suggesting lower resilience than those in other areas. Further research is needed to gain a better understanding of the changes in the bee gut microbiota between different areas and seasons by collecting samples at more different time points throughout the year. This will help us to understand how environmental stressors affect the gut microbial communities in these areas. However, it is clear by ours results that urbanization process and climate changes can alter the microbiota of *M. capixaba*.

References

1. Douglas AE. Multiorganismal insects: Diversity and function of resident microorganisms. Annu Rev Entomol. 2015;60: 17–34. doi:10.1146/annurev-ento-010814-020822
2. Akami M, Ren XM, Qi X, Mansour A, Gao B, Cao S, et al. Symbiotic bacteria motivate the foraging decision and promote fecundity and survival of *Bactrocera dorsalis* (Diptera: Tephritidae). BMC Microbiol. 2019;19: 1–13. doi:10.1186/s12866-019-1607-3
3. Liberti J, Engel P. The gut microbiota — brain axis of insects. Curr Opin Insect Sci. 2020;39: 6–13. doi:10.1016/j.cois.2020.01.004
4. Gould AL, Zhang V, Lamberti L, Jones EW, Obadia B, Korasidis N, et al. Microbiome interactions shape host fitness. Proc Natl Acad Sci U S A. 2018;115: E11951–E11960. doi:10.1073/pnas.1809349115
5. Kwong WK, Moran NA. Gut Microbial Communities of Social Bees. Physiol Behav. 2017;176: 139–148. doi:10.1038/nrmicro.2016.43.Gut
6. Kwong WK, Mancenido AL, Moran NA. Immune system stimulation by the native gut microbiota of honey bees. R Soc Open Sci. 2017;4: 1–9. doi:10.1098/rsos.170003
7. Wu Y, Zheng Y, Chen Y, Wang S, Chen Y, Hu F, et al. Honeybee (Apis mellifera) gut microbiota promotes host endogenous detoxification capability via regulation of P450 gene expression in the digestive tract. Microb Biotechnol. 2020;13: 1201–1212. doi:10.1111/1751-7915.13579
8. Engel P, Martinson VG, Moran NA. Functional diversity within the simple gut microbiota of the honeybee. PNAS. 2012;109. doi:10.1073/pnas.1202970109/-/DCSupplemental.www.pnas.org/cgi/doi/10.1073/pnas.1202970109
9. Powell JE, Martinson VG, Urban-mead K, Moran A. Routes of Acquisition of the Gut Microbiota of the Honeybee *Apis mellifera*. Appl Environ Microbiol. 2014;80: 7378–7387. doi:10.1128/AEM.01861-14
10. Michener CD. The Bees of the World. 2nd ed. The Johns Hopkins University Press; 2007.
11. Moure JS, Camargo JMF. *Melipona (Michmelia) capixaba*, uma nova espécie de Meliponinae (Hymenoptera, Apidar) do sudeste do Brasil. Rev Bras Zool. 1994;11: 289–296.
12. Resende HC, Fernandes-Salomão TM, Tavares MG, Campos L. Geographic distribution, key challenges, and prospects for the conservation of threatened stingless bee *Melipona capixaba* Moure and Camargo (Hymenoptera: Apidae: Meliponini). Sociobiology. 2014;61: 529–535. doi:10.13102/sociobiology.v61i4.529-535
13. BRASIL. Portaria MMA nº 148, de 7 de Julho de 2022. 2022. Available: https://www.icmbio.gov.br/cepsul/destaques-e-eventos/704-atualizacao-da-lista-oficial-das-especies-ameacadas-de-extincao.html
14. Luz CFP Da, Fernandes-Salomão TM, Lage LGA, Resende HC, Tavares MG, Campos LADO. Pollen sources for *Melipona capixaba* Moure & Camargo: An endangered Brazilian stingless bee. Psyche (Stuttg). 2011. doi:10.1155/2011/107303
15. Cerqueira AES, Hammer TJ, Moran NA, Santana WC, Kasuya MCM, Silva CC da. Extinction of anciently associated gut bacterial symbionts in a clade of stingless bees. ISME J. 2021; 2–5. doi:10.1038/s41396-021-01000-1
16. Haag KL, Caesar L, da Silveira Regueira-Neto M, de Sousa DR, Montenegro Marcelino V, de Queiroz Balbino V, et al. Temporal Changes in Gut Microbiota

Composition and Pollen Diet Associated with Colony Weakness of a Stingless Bee. Microb Ecol. 2022. doi:10.1007/s00248-022-02027-3
17. Sarton-Lohéac G, Nunes da Silva CG, Mazel F, Baud G, de Bakker V, Das S, et al. Deep Divergence and Genomic Diversification of Gut Symbionts of Neotropical Stingless Bees. Graf J, editor. mBio. 2023;14. doi:10.1128/mbio.03538-22
18. Martinson VG, Moy J, Moran NA. Establishment of characteristic gut bacteria during development of the honeybee worker. Appl Environ Microbiol. 2012;78: 2830–2840. doi:10.1128/AEM.07810-11
19. Rothman JA, Russell KA, Leger L, McFrederick QS, Graystock P. The direct and indirect effects of environmental toxicants on the health of bumblebees and their microbiomes: Impact of toxicants on bumblebee health. Proceedings of the Royal Society B: Biological Sciences. 2020;287. doi:10.1098/rspb.2020.0980
20. Motta EVS, Raymann K, Moran NA. Glyphosate perturbs the gut microbiota of honeybees. Proc Natl Acad Sci U S A. 2018;115: 10305–10310. doi:10.1073/pnas.1803880115
21. Powell JE, Carver Z, Leonard SP. Field-Realistic Tylosin Exposure Impacts Honeybee Microbiota and Pathogen Susceptibility, Which Is Ameliorated by Native Gut Probiotics. Microbiol Spectr. 2021;9.
22. Ludvigsen J, Rangberg A, Avershina E, Sekelja M, Kreibich C, Amdam G, et al. Shifts in the midgut/pyloric microbiota composition within a honeybee apiary throughout a season. Microbes Environ. 2015;30: 235–244. doi:10.1264/jsme2.ME15019
23. Hall MA, Brettell LE, Liu H, Nacko S, Spooner-Hart R, Riegler M, et al. Temporal changes in the microbiome of stingless bee foragers following colony relocation. FEMS Microbiol Ecol. 2021;97: 1–12. doi:10.1093/femsec/fiaa236
24. Alvares CA, Stape JL, Sentelhas PC, De Moraes Gonçalves JL, Sparovek G. Köppen's climate classification map for Brazil. Meteorologische Zeitschrift. 2013;22: 711–728. doi:10.1127/0941-2948/2013/0507
25. Varela-Martínez DA, González-Sálamo J, González-Curbelo MÁ, Hernández-Borges J. Quick, easy, cheap, effective, rugged, and safe (QUECHERS) extraction. Liquid-Phase Extraction. Elsevier; 2019. pp. 399–437. doi:10.1016/B978-0-12-816911-7.00014-1
26. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. 2016;13: 581–583. doi:10.1038/nmeth.3869
27. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. 2011. Available: http://www-huber.embl.de/users/an-
28. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. Nucleic Acids Res. 2013;41. doi:10.1093/nar/gks1219
29. Oksanen J, Simpson GL, Blanchet FG, Kindt R, Legendre P, Minchin PT, et al. vegan: Community Ecology Package. 2022.
30. Wickham H. ggplot2: Elegant Graphics for Data Analysis. ggplot2. Springer New York; 2016. doi:10.1007/978-0-387-98141-3
31. Liu H, Hall MA, Brettell LE, Wang J, Halcroft M, Nacko S, et al. Microbial diversity in stingless bee gut is linked to host wing size and influenced by the environment. J Invertebr Pathol. 2023;198. doi:10.1016/j.jip.2023.107909
32. Motta EVS, Moran NA. Impact of Glyphosate on the Honeybee Gut Microbiota: Effects of Intensity, Duration, and Timing of Exposure. mSystems. 2020;5. doi:10.1128/msystems.00268-20

33. Motta EVS, Mak M, De Jong TK, Powell JE, O'Donnell A, Suhr KJ, et al. Oral or topical exposure to glyphosate in herbicide formulation impacts the gut microbiota and survival rates of honeybees. Appl Environ Microbiol. 2020;86: 1–21. doi:10.1128/AEM.01150-20
34. Botina LL, Barbosa WF, Acosta JPL, Bernardes RC, Cortes JEQ, Pylro VS, et al. The impact of early-life exposure to three agrochemicals on survival, behavior, and gut microbiota of stingless bees (*Partamona helleri*). Environmental Science and Pollution Research. 2023;30: 70143–70158. doi:10.1007/s11356-023-27385-4
35. Hotchkiss MZ, Poulain AJ, Forrest JRK. Pesticide-induced disturbances of bee gut microbiotas. FEMS Microbiol Rev. 2022;46: 1–22. doi:10.1093/femsre/fuab056
36. Kakumanu ML, Reeves AM, Anderson TD, Rodrigues RR, Williams MA. Honeybee gut microbiome is altered by in-hive pesticide exposures. Front Microbiol. 2016;7. doi:10.3389/fmicb.2016.01255
37. Erban T, Vaclavikova M, Tomesova D, Halesova T, Hubert J. tau-Fluvalinate and other pesticide residues in honeybees before overwintering. Pest Manag Sci. 2019;75: 3245–3251. doi:10.1002/ps.5446
38. da Costa DM, Batista MC, de Brito AS, de Barros I, Teodoro AV. Rainfall, temperature, and vegetation type influence nesting by the oil-collecting bee *Centris (Hemisiella) tarsata* in Brazilian restinga. Apidologie. 2019;50: 811–820. doi:10.1007/s13592-019-00688-7
39. Lawson DA, Rands SA. The effects of rainfall on plant–pollinator interactions. Arthropod-Plant Interactions. Springer Netherlands; 2019. pp. 561–569. doi:10.1007/s11829-019-09686-z
40. Forsgren E, Olofsson TC, Vasquez A, Fries I. Novel lactic acid bacteria inhibiting Paenibacillus larvae in honeybee larvae. Apidologie. 2010;41: 99–108.
41. Chen J, Wang J, Zheng H. Characterization of *Bifidobacterium apousia* sp. nov., *Bifidobacterium choladohabitans* sp. nov., and *Bifidobacterium polysaccharolyticum* sp. nov., three novel species of the genus *Bifidobacterium* from honeybee gut. Syst Appl Microbiol. 2021;44. doi:10.1016/j.syapm.2021.126247
42. Butler È, Alsterfjord M, Olofsson TC, Karlsson C, Malmström J, Vásquez A. Proteins of novel lactic acid bacteria from *Apis mellifera mellifera*: An insight into the production of known extra-cellular proteins during microbial stress. BMC Microbiol. 2013;13. doi:10.1186/1471-2180-13-235
43. Knoll S, Pinna W, Varcasia A, Scala A, Cappai MG. The honey bee (Apis mellifera L., 1758) and the seasonal adaptation of productions. Highlights on summer to winter transition and back to summer metabolic activity. A review. Livestock Science. Elsevier B.V.; 2020. doi:10.1016/j.livsci.2020.104011
44. Freitas PVDX, Faquinello P, Arnhold E, Ferro DAC, Ferro RAC, Lacerda MLG, et al. Flight radius and climatic conditions affect the external activity of stingless bee *Melipona rufiventris* (Lepeletier, 1836). Brazilian Journal of Biology. 2023;83. doi:10.1590/1519-6984.275645
45. Xavier de Freitas PVD, da Silva IE, Faquinello P, Zanata RA, Arnhold E, e Silva-Neto C de M. External activity of the stingless bee *Melipona fasciculata* (Smith) kept in the Brazilian Cerrado. J Apic Res. 2022;61: 429–434. doi:10.1080/00218839.2020.1745436
46. Cornman RS, Tarpy DR, Chen Y, Jeffreys L, Lopez D, Pettis JS, et al. Pathogen webs in collapsing honey bee colonies. PLoS One. 2012;7. doi:10.1371/journal.pone.0043562
47. Kwong WK, Medina LA, Koch H, Sing K, Jia E, Soh Y, et al. Dynamic microbiome evolution in social bees. Sci Adv. 2017;3: 1–17. doi:10.1126/sciadv.1600513

48. Bleau N, Bouslama S, Giovenazzo P, Derome N. Dynamics of the honeybee (*Apis mellifera*) gut microbiota throughout the overwintering period in Canada. Microorganisms. 2020;8: 1–11. doi:10.3390/microorganisms8081146
49. de Sousa LP. Bacterial communities of indoor surface of stingless bee nests. PLoS One. 2021;16. doi:10.1371/journal.pone.0252933
50. Nogueira-Neto P. Vida e Criação de Abelhas Indígenas Sem Ferrão. 1997.
51. Keller A, McFrederick QS, Dharampal P, Steffan S, Danforth BN, Leonhardt SD. (More than) Hitchhikers through the network: The shared microbiome of bees and flowers. Current Opinion in Insect Science. Elsevier Inc.; 2021. pp. 8–15. doi:10.1016/j.cois.2020.09.007
52. Nguyen PN, Rehan SM. The effects of urban land use gradients on wild bee microbiomes. Front Microbiol. 2022;13. doi:10.3389/fmicb.2022.992660
53. Ramalho M. Stingless bees and mass flowering trees in the canopy of Atlantic Forest: a tight relationship. Acta Bot Brasilica. 2003;18.
54. Kešnerová L, Emery O, Troilo M, Liberti J, Erkosar B, Engel P. Gut microbiota structure differs between honeybees in winter and summer. ISME Journal. 2020;14: 801–814. doi:10.1038/s41396-019-0568-8
55. Jones JC, Fruciano C, Hildebrand F, Al Toufalilia H, Balfour NJ, Bork P, et al. Gut microbiota composition is associated with environmental landscape in honeybees. Ecol Evol. 2018;8: 441–451. doi:10.1002/ece3.3597
56. Tommasi N, Colombo B, Pioltelli E, Biella P, Casiraghi M, Galimberti A. Urban habitat fragmentation and floral resources shape the occurrence of gut parasites in two bumblebee species. Ecol Evol. 2023;13. doi:10.1002/ece3.10299
57. Ptaszyńska AA, Latoch P, Hurd PJ, Polaszek A, Michalska-Madej J, Grochowalski Ł, et al. Amplicon sequencing of variable 16s rrna from bacteria and its2 regions from fungi and plants, reveals honeybee susceptibility to diseases results from their forage availability under anthropogenic landscapes. Pathogens. 2021;10. doi:10.3390/pathogens10030381
58. McFrederick QS, Rehan SM. Wild Bee Pollen Usage and Microbial Communities Co-vary Across Landscapes. Microb Ecol. 2019;77: 513–522. doi:10.1007/s00248-018-1232-y
59. Nguyen PN, Rehan SM. Environmental Effects on Bee Microbiota. Microbial Ecology. Springer; 2023. pp. 1487–1498. doi:10.1007/s00248-023-02226-6
60. Marshman J, Blay-Palmer A, Landman K. Anthropocene crisis: Climate change, pollinators, and food security. Environments - MDPI. MDPI AG; 2019. doi:10.3390/environments6020022

Supplementary Material

FL19.02 - RELATÓRIO DE ENSAIO II	Revisão: 11 Emissão: 17/03/2023 Aprovado por: Luana Emitido por: Dilson
DOCUMENTO DO SISTEMA DE GESTÃO	

N° 2909-01 / LARP

Cliente: FUNARBE - Fundação Arthur Bernardes
CPF/CNPJ: 20.320.503/0001-51
Endereço: Av. PH Rolfs, s/n - Campus Universitário, Bairro -, CEP -, Viçosa/MG
Tipo de amostra: MEL
Identificação pelo cliente: VNI1
Data de recebimento: 14/06/2023 Data de análise: 16/08/2023
Método utilizado: Determinação de Resíduos de Pesticidas Empregando Método QuEChERS modificado e LC-MS/MS Procedimento: POP102 rev. 15

Compostos analisados	Limites do método	
	LOD (mg/kg)	LOQ (mg/kg)
acetamiprido, ametrina, azaconazol, azametifós, azinfós metílico, azoxistrobina, boscalida, bromofós metílico, bromuconazol, buprofezina, butóxido de piperonila, carbaril, carbendazim, carbofurano, carboxim, ciazofamida, cimoxanil, ciproconazol, clofentezina, clorantraniliprole, clorfenvinfós, clorimurom etílico, clorpirifós, clotianidina, cresoxim metílico, demeton-s-metil-sulfona, diazinona, diclorvós, diclosulam, dicrotofós, difenoconazol, diflubenzurom, dimetoato, dimoxistrobina, diurom, EPN, epoxiconazol, espinosade A, espinosade D, etiofencarbe, etiofencarbe sulfona, etofenpróxi, etoprofós, etoxissulfurom, fenamidona, fenamifós, fenarimol, fenazaquim, fenoxicarbe, fenpiroximato, fenpropimorfe, fentiona, fipronil, fluazafope-p-butílico, fluquinconazol, flusilazol, flutolanil, flutriafol, fosalona, fosmete, furatiocarbe, hexitiazóxi, imidacloprido, iprovalicarbe, malationa, mecarbam, mefosfolam, mepronil, metalaxil, metconazol, metidationa, metiocarbe, metiocarbe sulfóxido, metomil, metoxifenozida, metsulfurom metílico, miclobutanil, monocrotofós, monolinurom, nicossulfurom, nuarimol, oxamil, paraoxom etílico, pencicurom, penconazol, penoxsulam, picoxistrobina, piraclostrobina, pirazofós, piridabem, piridafentiona, piridato, pirimetanil, pirimicarbe, pirimifós etílico, pirimifós metílico, piriproxifeno, profenofós, prometrina, propanil, propargito, propiconazol, propoxur, quinoxifeno, quizalofope-p-etílico, saflufenacil, simazina, tebufenozida, tebufenpirade, tetraconazol, tiabendazol, tiacloprido, tiametoxam, tiobencarbe, tiodicarbe, tiofanato metílico, tolifluanida, triadimefom, triazofós, triciclazol, trifloxissulfurom, triflumizol, triflumurom, vamidotiona	0,001	0,003
aldicarbe, azinfós etílico, bitertanol, cianazina, clorpirifós metílico, deltametrina, forato, linurom, molinato, quinalfós, terbufós, tolclofós metílico, trifloxistrobina	0,002	0,005
fenhexamida, metribuzim, oxadixil	0,003	0,010

LOD = Limite de detecção do método; LOQ = Limite de quantificação do método

Resultados	Concentração (mg/kg)
Compostos analisados não foram detectados (< LOD).	

< LOQ = menor que o LOQ, ou seja, o composto está presente na amostra em nível de concentração que não pode ser quantificado pelo método.
Os resultados são apresentados para as amostras entregues no LARP pelo cliente.

 Data de emissão: 24/08/2023
Verificação: Cleusa Zanchin

Signatário Autorizado

Laboratório de Análises de Resíduos de Pesticidas – LARP
Campus UFSM, Rua E, Prédio 13D-LARP, CEP 97.105-900, Camobi, Santa Maria, RS Fone: (055) 3220-8011 e-mail: larp@ufsm.br

CAPÍTULO 3

Dimethoate is highly toxic for *Melipona* bees but does not affect their gut microbiome in sublethal doses

(Capítulo formatado de acordo com as normas da revista PLOS ONE)

Abstract

Stingless bees are native to tropical and subtropical regions, where they serve a crucial role in pollinating local ecosystems and crops. Anthropic actions, including the intensive use of pesticides, have led to a decline in bee populations worldwide. While research has mainly focused on the direct effects of agrochemicals on bees, especially honeybees, little is known about how these compounds may affect stingless bees, particularly in sublethal doses. This study aimed to explore the impact of dimethoate, an agrochemical, on the gut microbiota of two species of Brazilian stingless bees, *Melipona mondury* and *M. quadrifasciata*. The bees were fed different doses of dimethoate for six hours and observed for 96 hours. The groups with a more than 60% survival rate had their gut dissected and the total DNA extracted and sequenced for the bacterial 16S rRNA gene. Results showed that dimethoate was highly toxic to both species, with *M. mondury* being more sensitive to lower doses. Although the agrochemical did not significantly impact the gut microbiota of the bees compared to the control group that was fed sucrose, the beta diversity was significantly different for the bees of the control groups (field and sucrose), including the relative abundance of the main bacterial families Bifidobacteriaceae and Acetobacteraceae in both bee species. Dimethoate does not impact the bee gut microbiota in sublethal doses but the laboratory conditions impacted their microbiome.

Introduction

The decline in the number of pollinators worldwide is a major concern due to their crucial role in maintaining biodiversity and ensuring food security. Bees are the most important pollinators for both native and crop plants, helping to sustain tropical and subtropical ecosystems [1,2]. Several bee populations have been lost due to

human activities that involve land cover changes, land-use intensity, and climate change [3–7]. In the Neotropical region, the decline of stingless bee populations has been mainly associated with pesticide exposure, as they seem to be more susceptible to those compounds than honeybee [8–12]. These agrochemicals can also trigger sublethal effects, affecting the behavior, physiology, morphology, and gut microbiota of those insects, making them more susceptible to pathogens and diseases [13–16].

Extensive research has been devoted to comprehending the impact of pesticides on bees directly. However, it is still unclear how these chemical compounds affect the gut microbiota of bees, which is an important part of bee biology [17]. Eusocial bees harbor dense and specialized microbial communities in their gut, which play a crucial role in bee health [18], host behavior, memory [19], and social networks [20]. However, *Melipona* lacks members of the core microbiome of other Apidae and presents a core-like microbiota that comprises members of Lactobacillaceae, Bifidobacteriaceae, Acetobacteraceae, and Streptococcaceae families [21–23].

Agrochemicals can indirectly affect the bee's health in sublethal doses by disturbing its gut microbiome. Glyphosate, an organophosphorus herbicide, impacts the gut microbiota of honeybees and decreases their survival when exposed to an opportunistic pathogen [24,25]. Similarly, Spinosad, $CuSO_4$, and glyphosate also affect the gut microbiota of *Partamona helleri*, a stingless bee commonly found in Brazil [26,27]. Other agrochemicals, such as fipronil, coumaphos, chlorpyrifos, and thiamethoxam, also impact the core microbiota of *Apis mellifera*, which is the most studied bee species [28–30]. The impacts of pesticides in the *Melipona* gut microbial community are still unknown.

Organophosphorus pesticides (OPs) are widely used for agricultural and residential purposes, making them one of the most used pesticides worldwide. In the United States alone, about 36 million kilograms of OPs are used annually, with 75% of that amount being used in agriculture [31]. In Brazil, dimethoate is a popular OP used to control various insects like plant hoppers, mites, flies, and aphids on crops such as grain, fruit, and vegetables. In 2017, approximately 703 tons of this insecticide were sold in the country [32,33]. However, dimethoate is highly toxic to bees, with the level of toxicity depending on the bee species [12]. This pesticide works by inhibiting acetylcholinesterase (AChE) activity, which is an enzyme that usually stops the action of neurotransmitters at neuromuscular junctions, like those found at cholinergic

synapses. This inhibition results in continuous muscle contractions, ultimately leading to the insect's death [34]. In addition, sublethal doses of dimethoate negatively affect the survival of honeybee larvae to American foulbrood infection [35].

Stingless bees (Meliponini) are a group of eusocial corbiculate bees that are diverse in nature. They are mostly found in tropical and subtropical regions, with over 600 bee species, with 244 species found in Brazil alone [36]. *Melipona quadrifasciata* is widely distributed in eastern Brazil [37], while *M. mondury* is mainly found in Atlantic Forest fragments. Both species are ecologically and economically important to the Brazilian fauna but are endangered due to habitat loss and anthropic actions [38]. Beekeepers in Brazil have reported annual losses of their *M. quadrifasciata* colonies, which is related to a syndrome that occurs in late summer, leading to colony collapse [39,40]. The reasons for this are still unclear, but both genetic and environmental factors are responsible for this collapse, which may include contact with sublethal doses of pesticides in the months before the collapse happens [41]. It is also known that the microbiota of these bees changes around two months before the outbreak period [21]. These bees are highly sensitive to sublethal doses of agrochemicals such as imidacloprid, which is known to cause behavioral changes and impairment of walking [42]. Exposure to organophosphorus, carbamate, and pyrethroid pesticides also leads to alteration of caste differentiation, impairment of social behavior, and asymmetry in different stingless bee species [43,44].

There is currently limited knowledge of the sublethal effects of agrochemicals on stingless bees [12]. Additionally, there are few reports on the effects of these compounds on the gut microbiota of these bees [26,27]. To address this knowledge gap, this study aims to assess the sublethal effects of acute oral exposure to the organophosphate dimethoate on the gut microbiota composition of *M. quadrifasciata* and *M. mondury*. Our hypothesis posits that the gut microbiota of *M. quadrifasciata* and *M. mondury* are not affected by sublethal doses of dimethoate.

Methodology

Insects and mortality bioassay

Three colonies of *M. quadrifasciata* were sampled in the rural area of the municipality of Coimbra (Minas Gerais State, Brazil), and three colonies of *M. mondury*

were sampled in Mata do Paraiso, in the municipality of Viçosa (Minas Gerais State, Brazil). Foragers bees were collected at the entrance of the hives and anesthetized by exposing them to carbon dioxide for 5 s. The bees were then transferred to transparent plastic containers (500 mL) and kept without food for 1 hour to acclimatize them to the experimental conditions and encourage them to feed on the provided diet. The experiment was conducted as part of a study by Ribas et al. (2024) to evaluate the toxicity of dimethoate in various bee species in Brazil.

The commercial formulation of the insecticide dimethoate, Dimexion® (emulsifiable concentrate; 400 g/L of active ingredient, cyclohexanone: 308.0 g/L, xylene: 290.0 g/L, other ingredients: 83 g/L; FMC Química do Brasil Ltda, São Paulo, Brazil), was used for the experiment.

Bees were divided into groups of 10 per colony per treatment, resulting in a total of 30 bees per treatment. *M. mondury* was treated with doses of 0.003, 0.013, 0.025, 0.036 and 0.065 µg a.i/bee. Otherwise, *M. quadrifasciata* was treated with doses of 0.018, 0.025, 0.070, 0.118, and 0.233 µg a.i/bee. The commercial formulation of dimethoate was diluted in sucrose solution (50% w/w) and provided to the bees for 6 hours using 2 mL microtubes inserted in 500 mL polyethylene containers. The control group (sucrose control) was fed only with sucrose solution. Afterward, the bees were allowed to feed on sucrose solution *ad libitum* until the end of the experiment. Mortality was observed for 96 hours, and insects were considered dead when they were unable to walk [12,45]. The survival data is available in the work of Ribas et al. (2024), which was also used to construct the Kaplan-Meier survival curve for the present work using GraphPad Prism 10 (Fig. 1).

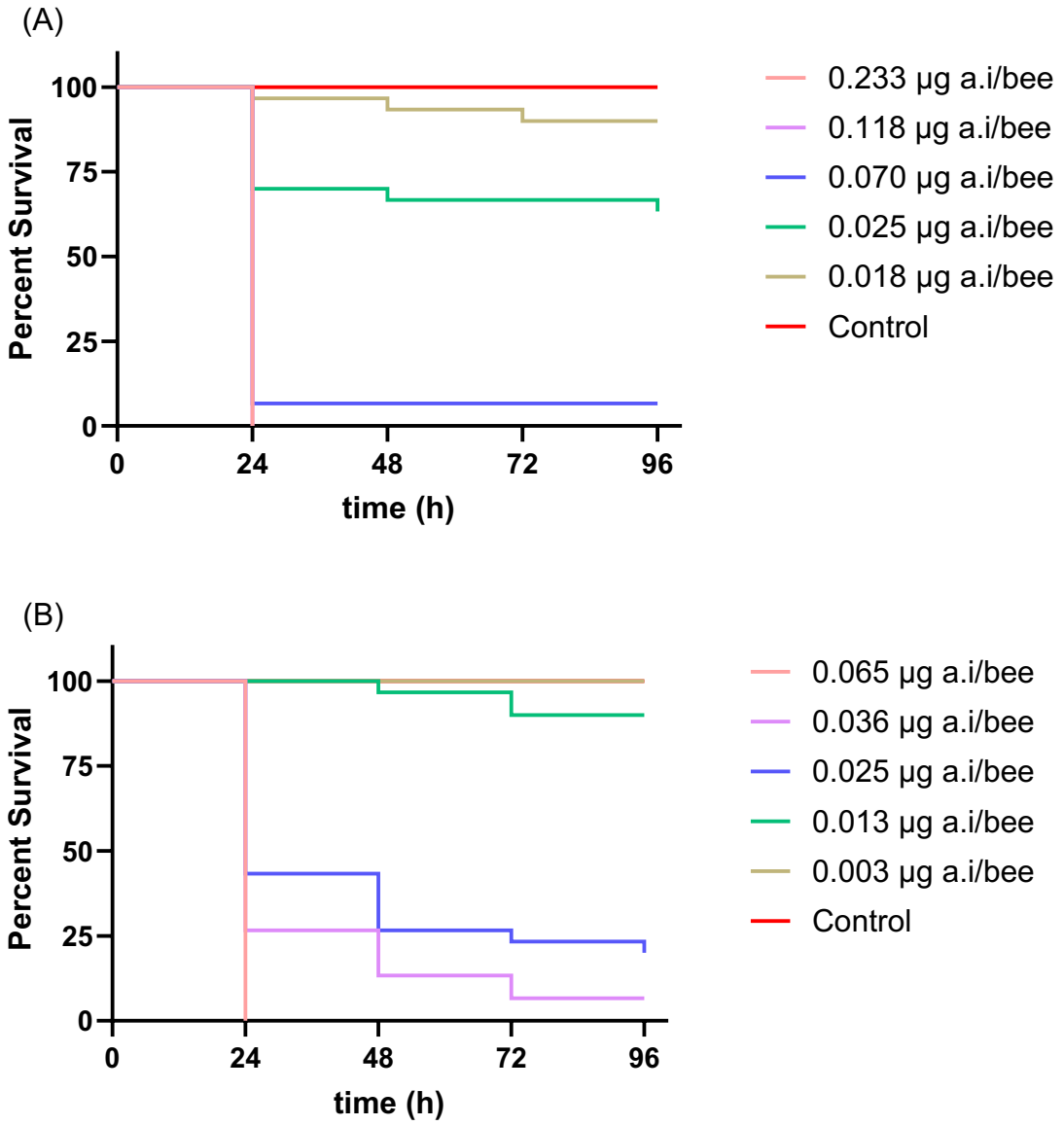


Figure 1. **Kaplan-Meier survival curves of *Melipona* bees orally exposed for 6 h to dimethoate.** The survival data was collected every 24 hours until 96 hours after the treatment. Each line represents a mean of the survival rate of the three colonies sampled for each group (A) *M. quadrifasciata*. (B) *M. mondury*.

DNA extraction, 16S rRNA gene sequencing, and data processing

M. quadrifasciata bees of sublethal groups (0.018 µg a.i/bee and 0.025 µg a.i/bee) and control groups, and *M. mondury* bees of sublethal groups (0.003 µg a.i/bee and 0.013 µg a.i/bee) and control groups were dissected (time = 96 h) with sterile forceps, and each sample was composed of an individual gut that had the total

genomic DNA extracted with the NucleoSpin Soil Kit (Macherey-Nagel), preceded by a proteinase K treatment for 2 hours at 56ºC [23]. As another control group, we sequenced bees before the exposure to dimethoate (time = 0, named field control). The DNA samples were submitted for 250 bp paired-end amplicon sequencing at Novogene Corporation Inc (Sacramento, CA, USA) using an Illumina NovaSeq 6000 System. The primer pair 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACNNGGGTATCTAAT) targeted the 16S rRNA V3-V4 regions.

The data was processed using the DADA2 package (version 1.28) [46] in R 4.3.1, following the pipeline available at https://benjjneb.github.io/dada2/tutorial.html. First, Cutadapt [47] was used to remove adapters. Then, the DADA2 pipeline was followed to filter, trim, de-replicate, denoise, and merge the paired-end sequences to construct the amplicon sequence variant (ASV) table. The taxonomy of the ASVs was assigned using a trained SILVA database (version 138.1 from November 2020) for bacteria [48].

The data analysis was conducted using three R packages: "mctoolsr" version 0.1.1.9, available at https://github.com/leffj/mctoolsr, "vegan" version 2.6-4 [49], and "ggplot2" version 3.4.2 [50]. The first step was to remove mitochondria, chloroplasts, and low-abundance ASVs (those with less than 100 total reads across all samples) from the dataset. The dataset was then rarefied to ensure an equal number of sequences per database. Alpha diversity was assessed using the Shannon and richness metrics. The relative abundances (beta diversity) were used to create a dissimilarity matrix based on the Bray-Curtis distance. The "Adonis" function was employed to perform the permutational multivariate analysis of variance (PERMANOVA) and Kruskal-Wallis test.

Results

Melipona bees are highly sensitive to the pesticide dimethoate. *M. quadrifasciata* bees were found to be affected by doses equal to or higher than 0.070 µg a.i/bee, and 100% of the bees exposed to 0.233 µg a.i/bee and 0.118 µg a.i/bee died within 24 hours (Fig 1A). After 96 hours, the survival rate of bees exposed to 0.070 µg a.i/bee was 6.7%. The group treated with 0.025 µg a.i/bee and 0.018 µg a.i/bee had survival rates of 63.3% and 90%, respectively. These groups were selected for gut microbiota diversity analysis.

Furthermore, *M. mondury* bees are even more vulnerable to dimethoate, with a dose of 0.065 µg a.i/bee causing the death of all bees within 24 hours and a dose of 0.036 µg a.i/bee resulting in a survival rate of 26.67% for the same period (Fig 1B). For gut microbiota analysis, doses of 0.013 µg a.i/bee and 0.003 µg a.i/bee were used, resulting in a survival rate of 96.67% and 100%, respectively, at the end of the experiment.

Bacterial diversity in the gut of Melipona bees treated with dimethoate

The evaluation the effect of sublethal doses of dimethoate on the bee gut microbiota was done in a total of 64 samples, including 28 *M. quadrifasciata* bees and 36 *M. mondury* bees. After processing in DADA2, the number of sequences per sample ranged from 10,922 to 152,863 reads. We removed the mitochondria, chloroplast, Eukaryota, and low-abundance ASV (<100), and retained 235 ASVs for further analysis. The ASVs were identified at the genus level, but for those identified only at the family or order level, we classified them as "Other" along with the family or order name.

No significant differences were found in the alpha diversity of bacterial communities of *M. mondury* and *M. quadrifasciata* between the controls and exposed bees (Figure S1).

Regarding the beta diversity, a non-metric multidimensional scaling (NMDS) analysis using the Bray-Curtis dissimilarity matrix (Figure 2) revealed that the gut community compositions of exposed and control bees are very close to each other for both species. The PERMANOVA analysis indicates that there are significant differences in the Bray-Curtis dissimilarity matrix between the groups. However, the pairwise PERMANOVA comparison between each treatment showed that only the field control and sucrose control are significantly different for *M. quadrifasciata* (Table S1). The microbiota of *M. mondury* bees treated with dimethoate in both sublethal doses also differ significantly from the field control group. Therefore, there is no significant difference between the bees treated with dimethoate and the sucrose control for either species.

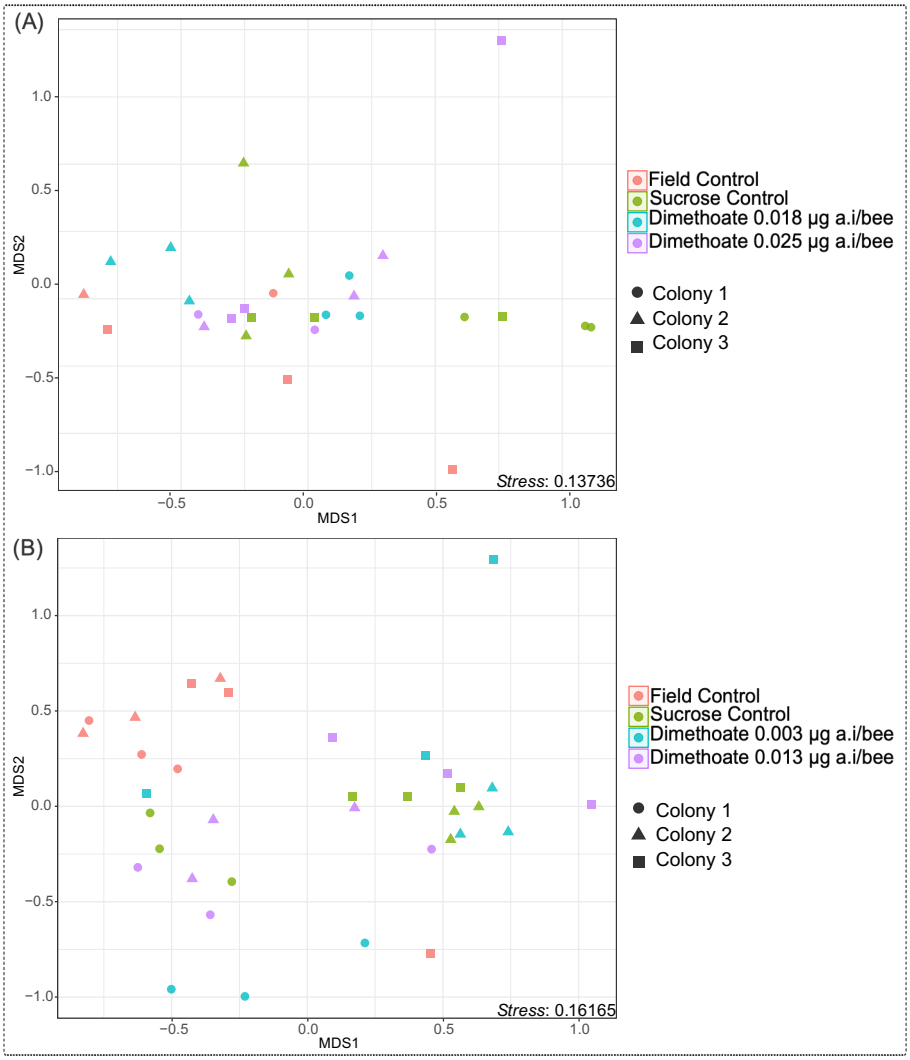


Figure 2. **Beta diversity of *Melipona* orally exposed to dimethoate. Non-metric multidimensional scaling (NMDS) of Bray-Curtis distances**. Each color represents one treatment or control group, and different shapes represent the hive where the bees were collected. (A) *M. quadrifasciata*. (B) *M. mondury*.

The samples, in general, indicated a low presence of bacteria from the Lactobacillaceae and Streptococcacea families (Figure S2). The most evident families in both bee species were Acetobacteraceae and Bifidobacteraceae (Figure S2), which were also confirmed in the analysis of the most abundant genera and ASVs in the samples (Figure 4 and Figure 5).

In *M. mondury*, there was a significant difference in the relative abundance of Bifidobacteriaceae and Acetobacteraceae between the field control bees and the groups analyzed in the experiment (Figure 3 C and D, S2 Table). However, this effect was not observed for *M. quadrifasciata* (Figure 3 A and B, S2 Table).

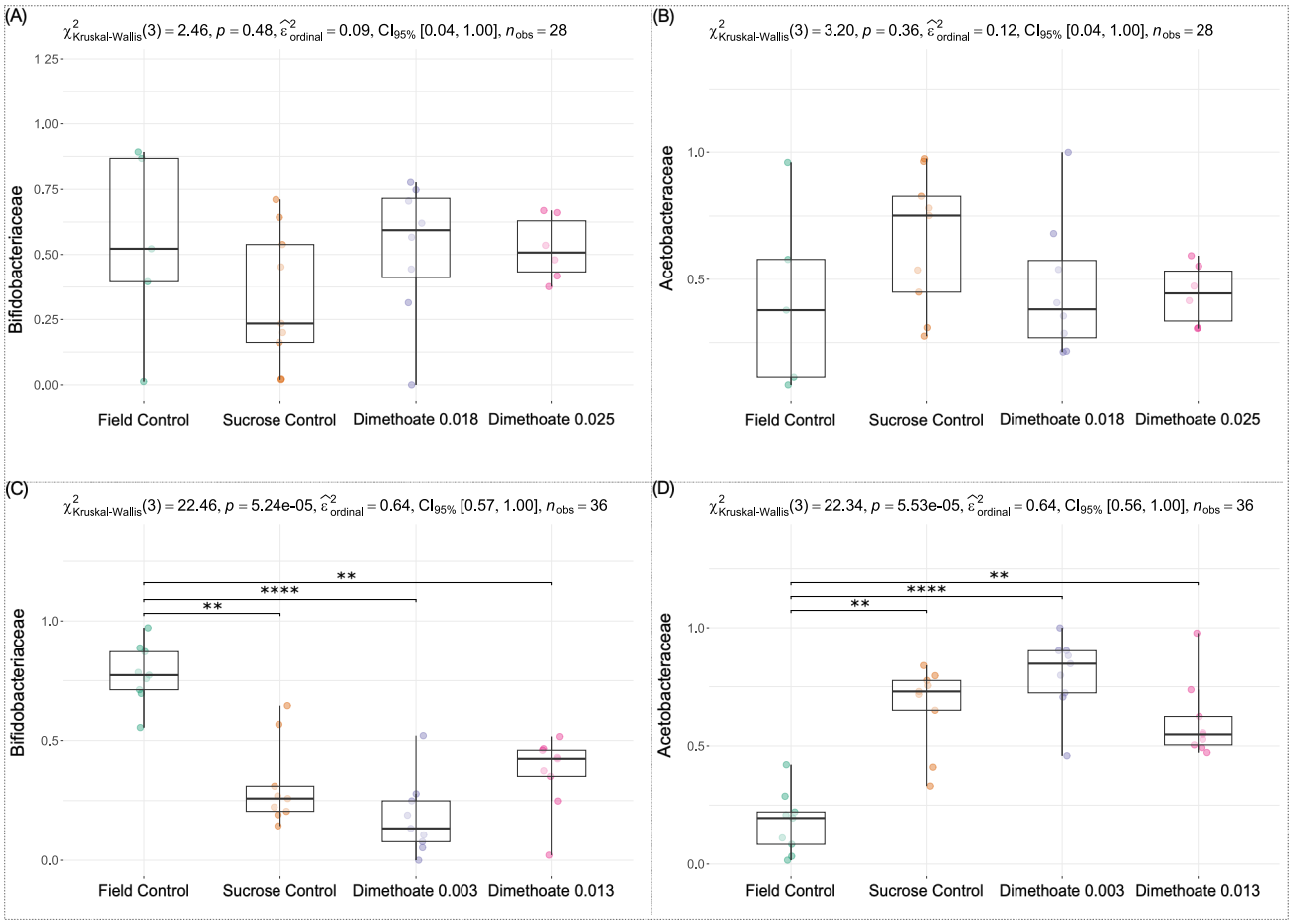


Figure 3. **Mean relative abundance of Bifidobacteriaceae and Acetobacteraceae of *Melipona* exposed to dimethoate and controls.** Kruskal Wallis test ($p < 0.05$) with post hoc Dunn tests. ** $p < 0.01$, **** $p < 0.0001$. (A) and (B) *M. quadrifasciata*. (C) and (D) *M. mondury*.

The microbiota of *M. quadrifasciata* did not present significant changes in the relative abundance of the most abundant bacterial genera across all the samples (Figure 4A, relative abundance > 0.001). However, bacteria of the Bifidobacteriaceae family, and *Bombella* were found to be the most dominant in all the samples, with their relative abundance varying depending on the group.

Otherwise, for *M. mondury*, the relative abundance of bacterial genera differed between the control bees and the treatment bees (Figure 4B). The field control bees had a higher relative abundance of Bifidobacteriaceae than the treatment bees, whereas the experiment bees had a higher abundance of *Neokomagataea* and *Bombella*, including the sucrose control. The bees from colony three also presented a

higher relative abundance of *Commensalibacter* in the field control and treatment of the dimethoate (0.003 µg a.i/bee), which differs from the bees of the other colonies used in the experiment.

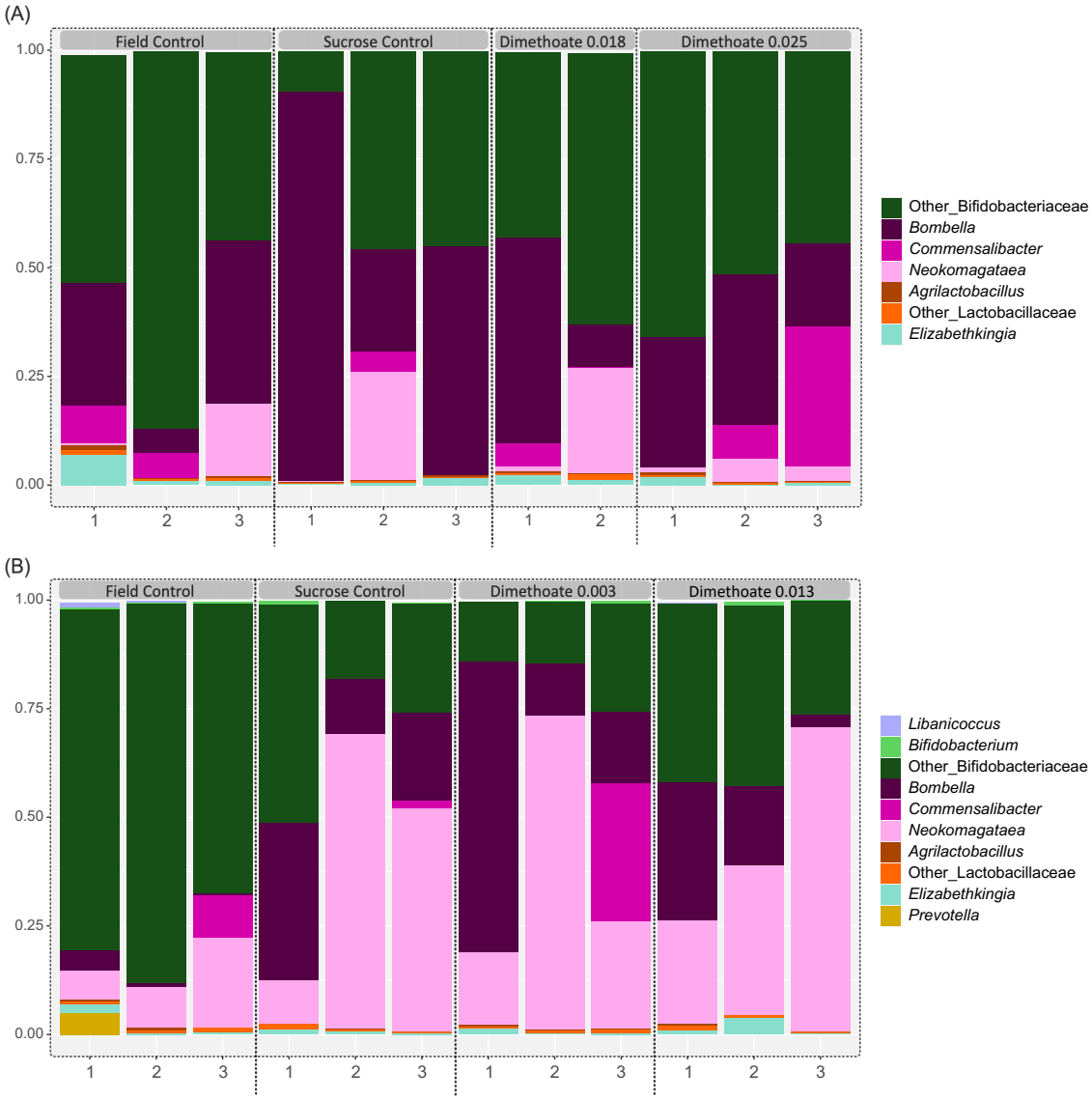


Figure 4. Mean relative abundance of bacterial genera (>0.001) in the gut microbiota of *Melipona* bees. The number on the x-axis corresponds to the colony number. 'Other_Bacteriaceae' and 'Other_Bifidobacteriaceae' refer to bacteria identified only at the family level; 'Other_Enterobacterales' refer to bacteria identified only at the order level. Dimethoate labels are expressed in µg a.i/bee of the insecticide. (A) *M. quadrifasciata anthidiodes*. (B) *M. mondury*.

The most abundant ASV in *M. quadrifasciata anthidioides* (Figure 5A), ASV4 (Bifidobacteriaceae), and ASV3 and ASV5 (*Bombella*), which, together with ASV13 (*Elizabethkingia*) were present in each sequenced bee. The ASV1 (Bifidobacteriaceae), even though it was one of the most abundant ASV, was not present in all the *M. quadrifasciata* bees sequenced. The bees from sucrose control, treatments of dimethoate 0.018 µg a.i/bee and 0.025 µg a.i/bee, had similar relative abundances of ASV1, ASV4, and ASV3. The ASV5 (*Bombella*) and ASV13 (*Elizabethkingia*) had lower abundances when compared to the other core ASVs.

Interestingly, for *M. mondury*, there was a significant change in the beta diversity of all the groups compared to the field control, which did not happen for the *M. quadrifasciata* bees (Table S1). ASV1 and ASV4, belonging to Bifidobacteriaceae, and ASV2 from *Neokomagataea* were the most abundant ASVs found in *M. mondury*. ASV3 (*Bombella*) had a low relative abundance in the field control and varied among the other treatments/colonies. The core ASVs in *M. mondury* treatments included ASV2 (*Neokomagataea*), ASV3 and ASV10 (*Bombella*), and ASV13 (*Elizabethkingia*). The latter two had the lowest abundance among the core ASVs. Although ASV1 was highly abundant, it was also not present in all sequenced *M. mondury* bee samples.

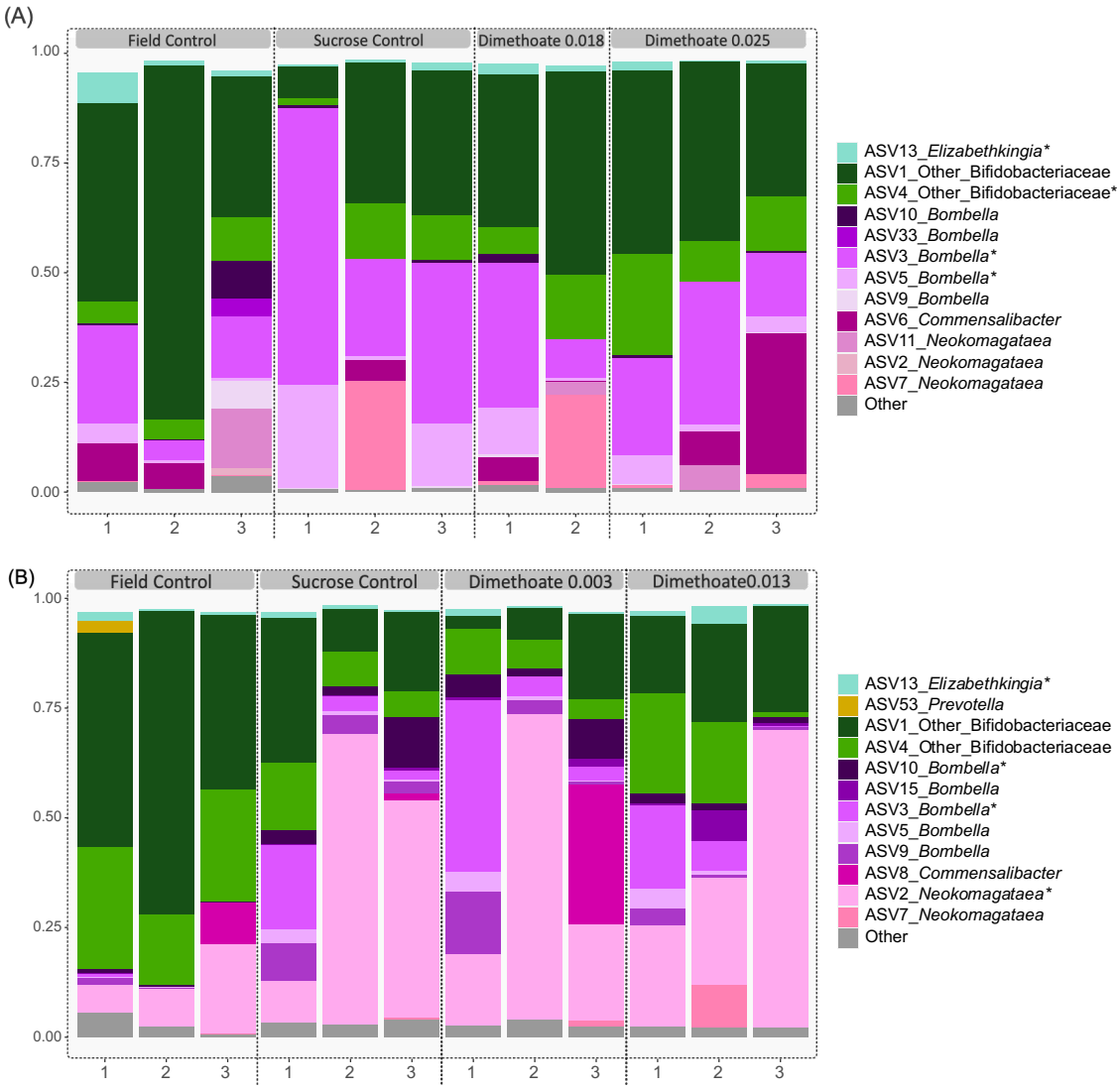


Figure 5. Mean relative abundance (>0.001) of most abundant ASVs in *Melipona* exposed to dimethoate and controls. *Core bacteria present in all bee samples. (A) *M. quadrifasciata*. (B) *M. mondury*.

Discussion

Dimethoate was a highly toxic insecticide for *Melipona* sp. However, it did not significantly affect their gut microbiota. Several studies had shown the toxicity of this organophosphorus insecticide in honeybees depending on the dose and route of exposure [30,51–53], with the standard LC_{50} value ranging from 0.10-0.35 µg a.i/bee according to OECD [45]. The mortality rate of dimethoate also varied among bee species [54]. *M. mondury* was more susceptible to the effects of dimethoate than *M. quadrifasciata*. The LC_{50} of *M. mondury* and *M. quadrifasciata* were 0.026 µg a.i/bee

and 0.034 µg a.i/bee, respectively [12]. This organophosphorus insecticide was rapidly absorbed in the animal gut [55], inhibiting the AChE activity in the brain of honeybees upon 24 h and 48 h of exposure, also inducing oxidative stress in those insects [52].

In this study, sublethal doses of dimethoate did not have a significant impact on the gut microbiota of bees. Compared to the control groups, the bacterial alpha diversity remained unaffected by the treatment with sublethal doses of dimethoate. Generally, various bacterial strains can produce enzymes that break down organophosphorus compounds, including dimethoate [56,57]. Past studies have established [58] that the gut microbiota of bees increases the production of enzymes responsible for the detoxification of pesticides and secondary metabolites of plants. Therefore, our results showed that the microbiota of *M. mondory and M. quadrifasciata* might possess resistance to sublethal doses of dimethoate by metabolizing it.

The microbiota of those bees was dominated by Bifidobacteriaceae and Acetobacteraceae. Interestingly, neither Lactobacillaceae nor Streptococcacea were one of the most abundant families in the bacterial community of the samples, which differed from the most abundant bacterial families in *Melipona* bees observed previously [21–23,59]. The bees were collected in late January/early February, and the local weather might had impacted the core microbiota of those insects. *M. quadrifasciata* from Southern Brazil also presented an increase in the relative abundance of Bifidobacteriaceae in the samples collected between January and February, followed by a decrease in the Lactobacillaceae abundance [21]. That fact was related to the availability of pollens in that area, which could also be an impacting factor for the bees in the present study.

The abundance of Acetobacteraceae and Bifidobacteriaceae differed significantly in *M. mondury*, but not in *M. quadrifasciata anthidiodes*. In *M. mondury*, the average relative abundance of Acetobacteraceae was lower in the field control group than in the treatment groups (sucrose control and dimethoate groups). Meanwhile, the mean relative abundance of Bifidobacteriaceae was higher in the field control group compared to the treatment groups. The increase in the abundance of Acetobacteraceae may be attributed to the consumption of a sucrose-rich diet. This family of bacteria is known for breaking down di-saccharides like sucrose into monosaccharides that they ferment into acetate and/or lactate [60]. They tend to thrive in environments that are high in sucrose, such as the crop of bees, also known as the

honey stomach [61]. Previous work has shown that when bees are fed with sucrose solution, there is an increase in the abundance of Acetobacteraceae family members, which can also impact the colonization of other bacteria in the bee gut [62].

The bacterial composition in both species of bees changed when comparing the field control and sucrose control bees. The bees in the study were not foraging for 96 hours, were under laboratory stress, and were not given pollen, all of which impacted their gut microbiota. *Melipona* bees rely on rich floral resources, such as pollen and nectar, to sustain their colony biomass year-round [63]. Pollen, in particular, is the primary source of lipid and protein for bees [64]. Hence, their microbial community's composition is affected by their consumption of pollen and nectar [21]. This means that forager bees, which experience different environmental factors and landscapes, exhibit varying abundances of dominant gut bacteria [65]. Honeybees fed with artificial diets have lower microbial diversity compared to pollen-fed bees, although none of the core lineages were lost in those treatments [66]. In another study, the total bacterial abundance and fermentative enzyme gene expression in honeybees' anterior rectum were reduced in a pollen-deficient diet. Conversely, pollen-fed bees showed heightened expression of the *Bifidobacterium hbd* gene (hydroxybutyryl-CoA dehydrogenase, *Bifidobacterium* specific bacterial metabolic gene) [67].

Conclusion

To summarize, while dimethoate is highly toxic to bees, exposure to sublethal doses of this pesticide has no significant effect on the gut bacterial community of *M. quadrifasciata* and *M. mondury*. During the study, it was observed that the bees' microbiota had a high relative abundance of bacteria from the Bifidobacteriaceae and Acetobacteraceae families but a very low abundance of bacteria from the Streptococcaceae and Lactobacillaceae families. It is also noted that the gut microbiota of the bees is sensitive to the laboratory and feeding conditions they are exposed to. Further research, mainly in proteomics, is needed to gain a better understanding of the role of the gut microbial community in protecting these *Melipona* bees against the toxic effects of dimethoate in sublethal doses.

References

1. Guimarães-Cestaro L, Martins MF, Martínez LC, Alves MLTMF, Guidugli-Lazzarini KR, Nocelli RCF, et al. Occurrence of virus, microsporidia, and pesticide residues in three species of stingless bees (Apidae: Meliponini) in the field. Science of Nature. 2020;107. doi:10.1007/s00114-020-1670-5
2. Jaffé R, Castilla A, Pope N, Imperatriz-Fonseca VL, Metzger JP, Arias MC, et al. Landscape genetics of a tropical rescue pollinator. Conservation Genetics. 2016;17: 267–278. doi:10.1007/s10592-015-0779-0
3. IPBES. The Assessment Report on Pollinators, Pollination and Food Production. 2016. Available: www.ipbes.net
4. Millard J, Outhwaite CL, Kinnersley R, Freeman R, Gregory RD, Adedoja O, et al. Global effects of land-use intensity on local pollinator biodiversity. Nat Commun. 2021;12. doi:10.1038/s41467-021-23228-3
5. Soroye P, Newbold T, Kerr J. Climate change contributes to widespread declines among bumble bees across continents. Science (1979). 2020. Available: https://www.science.org
6. Woodcock BA, Isaac NJB, Bullock JM, Roy DB, Garthwaite DG, Crowe A, et al. Impacts of neonicotinoid use on long-term population changes in wild bees in England. Nat Commun. 2016;7. doi:10.1038/ncomms12459
7. LeBuhn G, Vargas Luna J. Pollinator decline: what do we know about the drivers of solitary bee declines? Current Opinion in Insect Science. Elsevier Inc.; 2021. pp. 106–111. doi:10.1016/j.cois.2021.05.004
8. de Morais CR, Travençolo BAN, Carvalho SM, Beletti ME, Vieira Santos VS, Campos CF, et al. Ecotoxicological effects of the insecticide fipronil in Brazilian native stingless bees *Melipona scutellaris* (Apidae: Meliponini). Chemosphere. 2018;206: 632–642. doi:10.1016/j.chemosphere.2018.04.153
9. Castilhos D, Bergamo GC, Gramacho KP, Gonçalves LS. Bee colony losses in Brazil: a 5-year online survey. Apidologie. 2019;50: 263–272. doi:10.1007/s13592-019-00642-7
10. Jacob CR de O, Zanardi OZ, Malaquias JB, Souza Silva CA, Yamamoto PT. The impact of four widely used neonicotinoid insecticides on *Tetragonisca angustula* (Latreille) (Hymenoptera: Apidae). Chemosphere. 2019;224: 65–70. doi:10.1016/j.chemosphere.2019.02.105
11. Dorneles AL, de Souza Rosa A, Blochtein B. Toxicity of organophosphorus pesticides to the stingless bees *Scaptotrigona bipunctata* and *Tetragonisca fiebrigi*. Apidologie. 2017;48: 612–620. doi:10.1007/s13592-017-0502-x
12. Ribas A, Botina LL, Araújo R dos S, Vidigal ML, Alves BC da S, Martins GF. Exploring honey bee toxicological data as a proxy for assessing dimethoate sensitivity in stingless bees. Preprint. 2024. doi:10.21203/rs.3.rs-3861005/v1
13. Vanbergen AJ. A cocktail of pressures imperils bees. Nature. 2021;596.
14. Siviter H, Bailes EJ, Martin CD, Oliver TR, Koricheva J, Leadbeater E, et al. Agrochemicals interact synergistically to increase bee mortality. Nature. 2021;596: 389–392. doi:10.1038/s41586-021-03787-7
15. Motta EVS, Moran NA. Impact of Glyphosate on the Honey Bee Gut Microbiota: Effects of Intensity, Duration, and Timing of Exposure. mSystems. 2020;5. doi:10.1128/msystems.00268-20
16. Steele MI, Motta EVS, Gattu T, Martinez D, Moran NA. The Gut Microbiota Protects Bees from Invasion by a Bacterial Pathogen. Microbiol Spectr. 2021;9.

17. Hotchkiss MZ, Poulain AJ, Forrest JRK. Pesticide-induced disturbances of bee gut microbiotas. FEMS Microbiology Reviews. Oxford University Press; 2022. doi:10.1093/femsre/fuab056
18. Raymann K, Moran NA. The role of the gut microbiome in health and disease of adult honey bee workers. Curr Opin Insect Sci. 2018;26: 97–104. doi:10.1016/j.cois.2018.02.012
19. Zhang Z, Mu X, Cao Q, Shi Y, Hu X, Zheng H. Honeybee gut Lactobacillus modulates host learning and memory behaviors via regulating tryptophan metabolism. Nat Commun. 2022;13. doi:10.1038/s41467-022-29760-0
20. Liberti J, Kay T, Quinn A, Kesner L, Frank ET, Cabirol A, et al. The gut microbiota affects the social network of honeybees. Nat Ecol Evol. 2022. doi:10.1038/s41559-022-01840-w
21. Haag KL, Caesar L, da Silveira Regueira-Neto M, de Sousa DR, Montenegro Marcelino V, de Queiroz Balbino V, et al. Temporal Changes in Gut Microbiota Composition and Pollen Diet Associated with Colony Weakness of a Stingless Bee. Microb Ecol. 2022. doi:10.1007/s00248-022-02027-3
22. Sarton-Lohéac G, Nunes da Silva CG, Mazel F, Baud G, de Bakker V, Das S, et al. Deep Divergence and Genomic Diversification of Gut Symbionts of Neotropical Stingless Bees. Graf J, editor. mBio. 2023;14. doi:10.1128/mbio.03538-22
23. Cerqueira AES, Hammer TJ, Moran NA, Santana WC, Kasuya MCM, Silva CC da. Extinction of anciently associated gut bacterial symbionts in a clade of stingless bees. ISME J. 2021; 2–5. doi:10.1038/s41396-021-01000-1
24. Motta EVS, Raymann K, Moran NA. Glyphosate perturbs the gut microbiota of honey bees. Proc Natl Acad Sci U S A. 2018;115: 10305–10310. doi:10.1073/pnas.1803880115
25. Motta EVS, Mak M, De Jong TK, Powell JE, O'Donnell A, Suhr KJ, et al. Oral or topical exposure to glyphosate in herbicide formulation impacts the gut microbiota and survival rates of honey bees. Appl Environ Microbiol. 2020;86: 1–21. doi:10.1128/AEM.01150-20
26. Botina LL, Vélez M, Barbosa WF, Mendonça AC, Pylro VS, Tótola MR, et al. Behavior and gut bacteria of *Partamona helleri* under sublethal exposure to a bioinsecticide and a leaf fertilizer. Chemosphere. 2019;234: 187–195. doi:10.1016/j.chemosphere.2019.06.048
27. Botina LL, Barbosa WF, Acosta JPL, Bernardes RC, Cortes JEQ, Pylro VS, et al. The impact of early-life exposure to three agrochemicals on survival, behavior, and gut microbiota of stingless bees (*Partamona helleri*). Environmental Science and Pollution Research. 2023;30: 70143–70158. doi:10.1007/s11356-023-27385-4
28. Paris L, Peghaire E, Moné A, Diogon M, Debroas D, Delbac F, et al. Honeybee gut microbiota dysbiosis in pesticide/parasite co-exposures is mainly induced by *Nosema ceranae*. J Invertebr Pathol. 2020;172. doi:10.1016/j.jip.2020.107348
29. Rouzé R, Moné A, Delbac F, Belzunces L, Blot N. The honeybee gut microbiota is altered after chronic exposure to different families of insecticides and infection by *Nosema ceranae*. Microbes Environ. 2019;34: 226–233. doi:10.1264/jsme2.ME18169
30. Yang Y, Ma S, Yan Z, Liu F, Diao Q, Dai P. Effects of three common pesticides on survival, food consumption and midgut bacterial communities of adult workers *Apis cerana* and *Apis mellifera*. Environmental Pollution. 2019;249: 860–867. doi:10.1016/j.envpol.2019.03.077

31. Miodovnik A. Prenatal exposure to industrial chemicals and pesticides and effects on neurodevelopment. Encyclopedia of Environmental Health. Elsevier; 2019. pp. 342–352. doi:10.1016/B978-0-12-409548-9.11008-5
32. Landau EC, da Silva GA, Matrangolo WJ, Pedrosa MW. Agricultura Orgânica. 1st ed. In: Landau EC, da Silva GA, Moura L, Hirsch A, Guimarães DP, editors. Dinâmica da Produção Agropecuária e da Paisagem Natural no Brasil nas Últimas Décadas. 1st ed. Brasília: Embrapa; 2020.
33. Dissanayake KN, Chou RCC, Thompson A, Margetiny F, Davie C, McKinnon S, et al. Impaired neuromuscular function by conjoint actions of organophosphorus insecticide metabolites omethoate and cyclohexanol with implications for treatment of respiratory failure. Clin Toxicol. 2021;59: 1239–1258. doi:10.1080/15563650.2021.1916519
34. Mohan Kumar H, Pannu AK, Kumar S, Sharma N, Bhalla A. Magnesium sulfate in organophosphorus compound poisoning: A prospective open-label clinician-initiated intervention trial with historical controls. Int J Crit Illn Inj Sci. 2022;12: 33–37. doi:10.4103/ijciis.ijciis_67_21
35. López JH, Krainer S, Engert A, Schuehly W, Riessberger-Gallé U, Crailsheim K. Sublethal pesticide doses negatively affect survival and the cellular responses in American foulbrood-infected honeybee larvae. Sci Rep. 2017;7. doi:10.1038/srep40853
36. Roubik DW. Stingless Bee (Apidae: Apinae: Meliponini) Ecology. Annual Reviews of Entomology. 2023;68: 231–256. doi:10.1146/annurev-ento-120120
37. Batata-Filho H, Melo GAR, Waldschimdt AM, Campos LAI, Fernandes-Salomão TM. Geographic distribution and spatial differentiation in the color pattern of abdominal stripes of the Neotropical stingless bee *Melipona quadrifasciata* (Hymenoptera: Apidae). Zoologia. 2009;2: 213–219.
38. Viana JL, De Abreu Cerqueira Sousa H, De Oliveira Alves RM, Pereira DG, Silva JC, Da Paixão JF, et al. Bionomics of *Melipona mondury* Smith 1863 (Hymenoptera: Apidae, meliponini) in relation to its nesting behavior. Biota Neotrop. 2015;15. doi:10.1590/1676-06032015009714
39. Díaz S, de Souza Urbano S, Caesar L, Blochtein B, Sattler A, Zuge V, et al. Report on the microbiota of *Melipona quadrifasciata* affected by a recurrent disease. J Invertebr Pathol. 2017;143: 35–39. doi:10.1016/j.jip.2016.11.012
40. Caesar L, Cibulski SP, Canal CW, Blochtein B, Sattler A, Haag KL. The virome of an endangered stingless bee suffering from annual mortality in Southern Brazil. Journal of General Virology. 2019;100: 1153–1164. doi:10.1099/jgv.0.001273
41. Caesar L, Lopes AMC, Radaeski JN, Bauermann SG, Konzen ER, Pombert JF, et al. Longitudinal survey reveals delayed effects of low gene expression on stingless bee colony health. J Apic Res. 2022;61: 654–663. doi:10.1080/00218839.2021.1962123
42. van Tomé H V., Martins GF, Lima MAP, Campos LAO, Guedes RNC. Imidacloprid-induced impairment of mushroom bodies and behavior of the native stingless bee *Melipona quadrifasciata anthidioides*. PLoS One. 2012;7. doi:10.1371/journal.pone.0038406
43. Dos Santos CF, Acosta AL, Dorneles AL, Dos Santos PDS, Blochtein B. Queens become workers: Pesticides alter caste differentiation in bees. Sci Rep. 2016;6. doi:10.1038/srep31605
44. Prado-Silva A, Nunes LA, dos Santos JM, Affonso PRA de M, Waldschmidt AM. Morphogenetic Alterations in *Melipona quadrifasciata anthidioides* (Hymenoptera: Apidae) Associated with Pesticides. Arch Environ Contam Toxicol. 2018;74: 627–632. doi:10.1007/s00244-018-0509-y

45. OECD. Test no. 213: Honeybees Acute Oral Toxicity Test. OECD Guidelines for the Testing of Chemicals. 1998.
46. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. 2016;13: 581–583. doi:10.1038/nmeth.3869
47. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. 2011. Available: http://www-huber.embl.de/users/an-
48. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. Nucleic Acids Res. 2013;41. doi:10.1093/nar/gks1219
49. Oksanen J, Simpson GL, Blanchet FG, Kindt R, Legendre P, Minchin PT, et al. vegan: Community Ecology Package. 2022.
50. Wickham H. ggplot2: Elegant Graphics for Data Analysis. ggplot2. Springer New York; 2016. doi:10.1007/978-0-387-98141-3
51. Thompson H, Cione A, Santos G. Is there a difference in the sensitivity of Africanised and European honeybees to thiamethoxam or dimethoate? Environmental Pollution. 2023;334. doi:10.1016/j.envpol.2023.122230
52. Christen V, Joho Y, Vogel M, Fent K. Transcriptional and physiological effects of the pyrethroid deltamethrin and the organophosphate dimethoate in the brain of honey bees (*Apis mellifera*). Environmental Pollution. 2019;244: 247–256. doi:10.1016/j.envpol.2018.10.030
53. Abay Z, Bezabeh A, Gela A, Tassew A. Evaluating the Impact of Commonly Used Pesticides on Honeybees (Apis mellifera) in North Gonder of Amhara Region, Ethiopia. J Toxicol. 2023;2023. doi:10.1155/2023/2634158
54. Uhl P, Franke LA, Rehberg C, Wollmann C, Stahlschmidt P, Jeker L, et al. Interspecific sensitivity of bees towards dimethoate and implications for environmental risk assessment. Sci Rep. 2016;6. doi:10.1038/srep34439
55. Kanungo D, Moretto A. Demithoate/Omethoate. In: FAO, WHO, editors. Pesticide residues in food - 2019. Geneva; 2019. pp. 239–252. Available: http://apps.who.int/bookorders.
56. Ahmad S, Pinto AP, Hai FI, Badawy METI, Vazquez RR, Naqvi TA, et al. Dimethoate residues in Pakistan and mitigation strategies through microbial degradation: a review. Environmental Science and Pollution Research. Springer Science and Business Media Deutschland GmbH; 2022. pp. 51367–51383. doi:10.1007/s11356-022-20933-4
57. Singh BK, Walker A. Microbial degradation of organophosphorus compounds. FEMS Microbiology Reviews. 2006. pp. 428–471. doi:10.1111/j.1574-6976.2006.00018.x
58. Wu Y, Zheng Y, Chen Y, Wang S, Chen Y, Hu F, et al. Honey bee (*Apis mellifera*) gut microbiota promotes host endogenous detoxification capability via regulation of P450 gene expression in the digestive tract. Microb Biotechnol. 2020;13: 1201–1212. doi:10.1111/1751-7915.13579
59. Figueroa LL, Maccaro JJ, Krichilsky E, Yanega D, McFrederick QS, Colleen Cavanaugh EM. Why Did the Bee Eat the Chicken? Symbiont Gain, Loss, and Retention in the Vulture Bee Microbiome. mBio. 2021;12. Available: https://journals.asm.org/journal/mbio
60. Lee FJ, Rusch DB, Stewart FJ, Mattila HR, Newton ILG. Saccharide breakdown and fermentation by the honey bee gut microbiome. Environ Microbiol. 2015;17: 796–815. doi:10.1111/1462-2920.12526
61. Corby-Harris V, Snyder LA, Schwan MR, Maes P, McFrederick QS, Anderson KE. Origin and effect of Alpha 2.2 Acetobacteraceae in honey bee larvae and description

of *Parasaccharibacter apium* gen. nov., sp. nov. Appl Environ Microbiol. 2014;80: 7460–7472. doi:10.1128/AEM.02043-14
62. Taylor MA, Robertson AW, Biggs PJ, Richards KK, Jones DF, Parkar SG. The effect of carbohydrate sources: Sucrose, invert sugar and components of mānuka honey, on core bacteria in the digestive tract of adult honey bees (*Apis mellifera*). PLoS One. 2019;14. doi:10.1371/journal.pone.0225845
63. Ramalho M. Stingless bees and mass flowering trees in the canopy of Atlantic Forest: a tight relationship. Acta Bot Brasilica. 2003;18.
64. Manning R. Fatty acids in pollen: a review of their importance for honey bees. Bee World. 2001;82.
65. Jones JC, Fruciano C, Hildebrand F, Al Toufalilia H, Balfour NJ, Bork P, et al. Gut microbiota composition is associated with environmental landscape in honey bees. Ecol Evol. 2018;8: 441–451. doi:10.1002/ece3.3597
66. Powell JE, Lau P, Rangel J, Arnott R, De Jong T, Moran NA. The microbiome and gene expression of honey bee workers are affected by a diet containing pollen substitutes. PLoS One. 2023;18. doi:10.1371/journal.pone.0286070
67. Ricigliano VA, Anderson KE. Probing the honey bee diet-microbiota-host axis using pollen restriction and organic acid feeding. Insects. 2020;11. doi:10.3390/insects11050291

Supplementary material

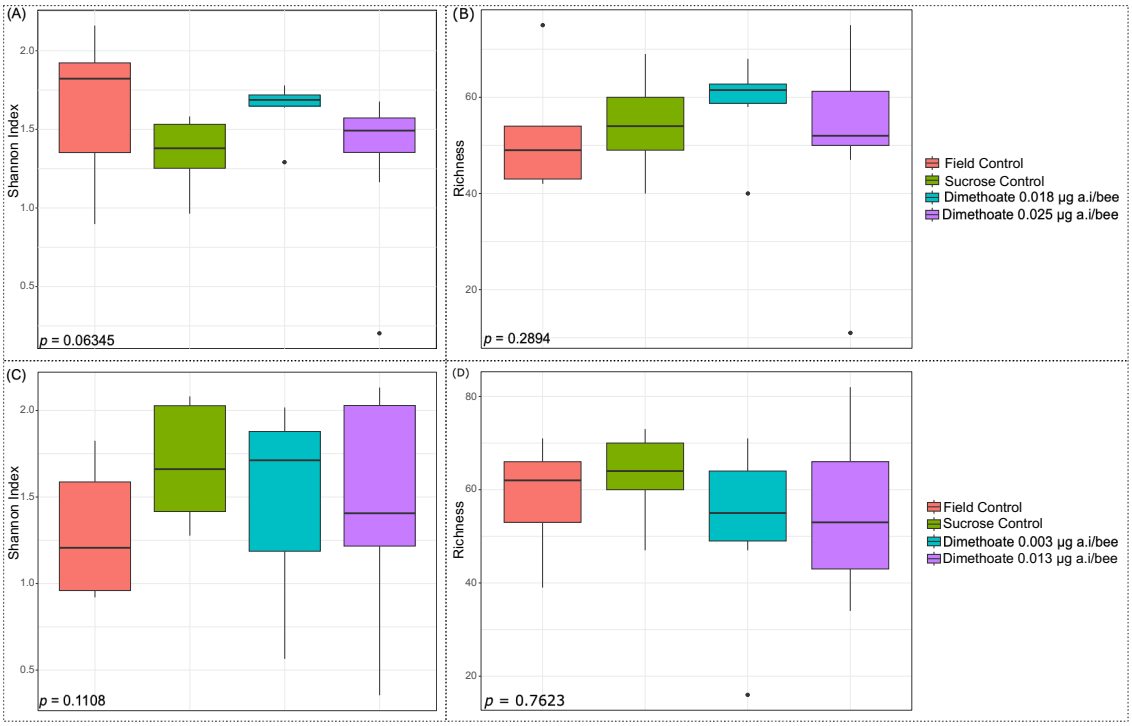
S1 Table. Pairwise comparison of the gut bacterial community of *Melipona* exposed to sublethal doses of dimethoate. Analysis was performed using PERMANOVA tests on the Bray-Curtis dissimilarity matrix ($p < 0.05$).

Source	Df	Sum of Squares	R2	F	Pr (>F)
M. quadrifasciata					
Field Control x Sucrose Control	1	0.35148	0.20723	3.13684	0.01798
Field Control x Dimethoate 0.018	1	0.18311	0.14035	1.46949	0.19480
Field Control x Dimethoate 0.025	1	0.14727	0.07782	0.92837	0.48151
Sucrose Control x Dimethoate 0.018	1	0.16228	0.09063	1.49506	0.17482
Sucrose Control x Dimethoate 0.025	1	0.20034	0.14541	2.21206	0.10589
Dimethoate 0.018 x Dimethoate 0.025	1	0.08928	0.06404	0.82111	0.57442
All groups	3	0.75332	0.18896	1.86395	0.03996
M. mondury					
Field Control x Sucrose Control	1	0.48392	0.27881	6.18561	0.00099
Field Control x Dimethoate 0.013	1	0.65790	0.25159	5.37872	0.00099
Field Control x Dimethoate 0.003	1	1.25896	0.34076	8.27055	0.00199
Sucrose Control x Dimethoate 0.013	1	0.06736	0.04785	0.80410	0.51948
Sucrose Control x Dimethoate 0.003	1	0.13523	0.07757	1.34549	0.21378
Dimethoate 0.003 x Dimethoate 0.013	1	0.13860	0.06792	1.16594	0.29370
All groups	3	1.62349	0.26466	3.83920	0.00099

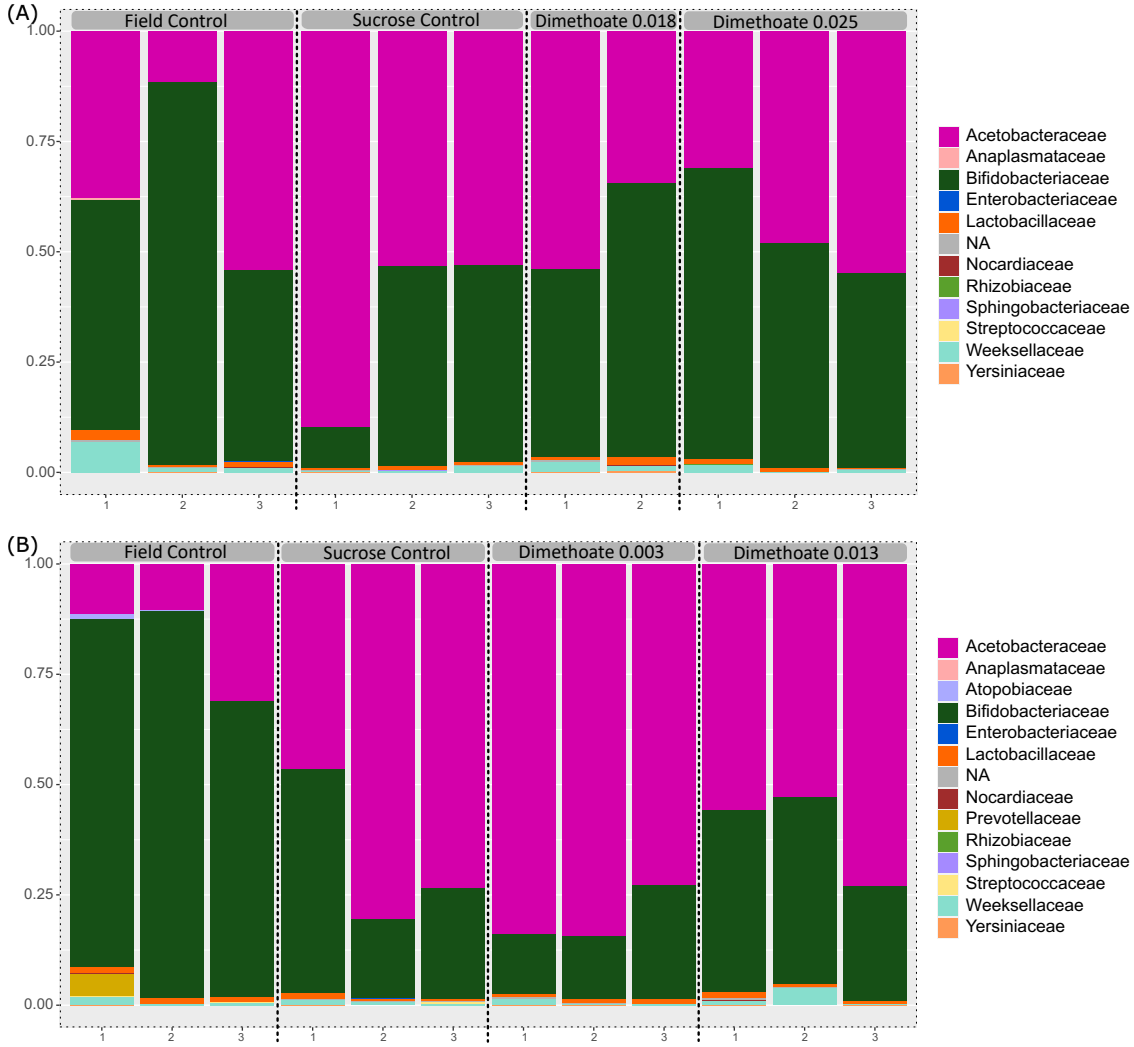
Dimethoate labels are expressed in µg a.i/bee of the insecticide.

S2 Table. Pairwise Kruskal-Wallis test with post hoc Dunn test ($p < 0.05$) for the mean relative abundance of Bifidobacteriaceae and Acetobacteraceae.

Pairwise comparison	$p_{\text{-adj.}}$ Bifidobacteriaceae	$p_{\text{-adj.}}$ Acetobacteraceae
M. quadrifasciata		
Fiel Control x Sucrose Control	0.60	0.44
Field Control x Dimethoate 0.018	0.98	0.84
Field Control x Dimethoate 0.025	0.98	0.84
Sucrose Control x Dimethoate 0.018	0.60	0.44
Sucrose Control x Dimethoate 0.025	0.60	0.51
Dimethoate 0.018 x Dimethoate 0.025	0.98	0.84
M. mondury		
Field Control x Sucrose Control	2.79e-03	2.57e-03
Field Control x Dimethoate 0.003	2.71e-05	3.01e-05
Field Control x Dimethoate 0.013	0.01	0.01
Sucrose Control x Dimethoate 0.003	0.24	0.26
Sucrose Control x Dimethoate 0.013	0.61	0.59
Dimethoate 0.003 x Dimethoate 0.013	0.11	0.12



S1 Figure. Bacterial alpha diversity expressed by Shannon Index and Richness of *Melipona* orally exposed to dimethoate. Kruskal Wallis test ($p < 0.05$). (A) Bacterial Shannon Index and (B) Observed bacterial richness in *M. quadrifasciata* samples. (C) Bacterial Shannon Index and (D) Observed bacterial richness in *M. mondury* samples.



S2 Figure. Mean relative abundance of bacterial families in the gut microbiota of *Melipona* bees. The number on the x-axis corresponds to the colony number. Dimethoate labels are expressed in µg a.i/bee of the insecticide (A) *Melipona quadrifasciata*. (B) *Melipona mondury*.

4. CONCLUSÃO GERAL

A microbiota *core* das abelhas do gênero *Melipona* difere das outras abelhas eusociais. Nossos dados, em todos os capítulos, confirmam a ausência de *Snodgrassella* e *Gilliamella* nessas abelhas, bem como a aquisição de novos simbiontes, como *Floricoccus* e *Apilactobacillus*. A descrição da diversidade microbiana em cada parte do intestino de *M. quadrifasciata* revela que novos simbiontes predominam no íleo dessas abelhas, indicando uma substituição dos microrganismos presentes em outras abelhas eusociais e ausentes em *Melipona*.

A comunidade bacteriana intestinal em *Melipona* apresenta diferenças em relação à paisagem e à sazonalidade. *M. capixaba* criadas em ambientes urbanos exibem menor diversidade microbiana em comparação àquelas criadas em outros ambientes. A microbiota dessas abelhas também sofre alterações significativas em relação às estações do ano. Esses fatores podem indicar desafios no estudo da microbiota intestinal de *Melipona*, uma vez que elas estão susceptíveis às variáveis ambientais e fatores antrópicos. Estudos futuros são necessários para compreender os impactos das mudanças ambientais na microbiota desses insetos, com um esforço amostral mais amplo e coletas durante todos os meses do ano, a fim de entender como essas alterações podem afetar a saúde dos indivíduos e das colmeias em geral.

Por fim, este trabalho também mostra que apesar do dimetoato ser altamente tóxico para *Melipona* spp., concentrações subletais desse agroquímico não afetam significativamente a microbiota intestinal de *M. mondury* e *M. quadrifasciata*. Curiosamente, os dados indicam uma alteração na composição microbiana intestinal das abelhas submetidas às condições laboratoriais, com um aumento na abundância de Acetobacteraceae e uma diminuição na abundância de Bifidobacteriaceae em relação ao grupo controle de campo. Esses resultados corroboram com os resultados listados anteriormente, em que fatores ambientais e estresse impactam a composição microbiana das abelhas do gênero *Melipona*.

Novos estudos envolvendo técnicas de isolamento e sequenciamento do genoma completo são necessários para uma melhor identificação e caracterização dos microrganismos simbionte, bem como os seus benefícios para a saúde das abelhas e das colmeias em geral.